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**A STUDY OF THE CELL ENVELOPE OF
HALOBACTERIUM SALINARIUM**

**A thesis submitted in partial fulfilment of the requirements
for the award of the degree of Doctor of Philosophy of the
University of Warwick.**

I wish to thank Professors V. M. Clark and G. M. Woodruff for
providing the facilities necessary for the research and Dr. J. H. Drenth for
his guidance.

School of Molecular Sciences

September, 1969



PREFACE

The work described in this thesis was carried out in the School of Molecular Sciences of the University of Warwick during the period October 1966 to July 1969. Except where otherwise stated, it is the author's original work, and has not been submitted for a degree at any other University.

I wish to thank Professors V.M. Clark and T.C. Waddington for providing the facilities necessary for the research and Dr. Jim Stevenson for continued guidance and encouragement.

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ABBREVIATIONS

Abbreviations listed in Policy of the Journal and Instructions to Authors (Biochemical Society) are used, where relevant, undefined. Additional abbreviations are as follows :

SDS sodium dodecyl sulphate

FRACTION CODE

For clarity, membrane fractions have been given a letter code, and are redefined at first mention in the text. The following is a general guide :

CEFB	cell envelope fragment bags
NM	crude, nuclease-treated membrane-rich fraction
R	purified membrane, free from amino sugar and nucleotide
RP	protein released with nucleotide from NM
RU	residual membrane fraction after treatment of R with urea
RUP	protein fragments released from R by treatment with urea
P	various protein fragments released from R in the absence of urea
SP	structural protein

ABSTRACT

The plasma membrane of Halobacterium salinarium, strain 1, an extremely halophilic bacterium, has been isolated and characterised. The cell envelope fraction (CEFB) was isolated from a cell homogenate by differential centrifugation. After dialysing the cell envelope fraction against distilled water and treating with nucleases, a fairly pure preparation of plasma membranes (NM) was obtained by centrifuging. The membrane-containing fraction was purified by gel filtration on Agarose, which yielded a purified membrane fraction (R) and a protein-nucleotide fraction (RP). A small molecular weight protein fraction P was separated from the purified membrane fraction either by gel filtration on Sephadex in a buffer containing phosphate and fluoride ions or by ultrafiltration. Protein fractions RUP_1 and RUP_2 were separated from the purified membrane by gel filtration on Agarose in the presence of 6 M urea. The remaining membrane-containing fraction, which was eluted in the void volume of the urea-Agarose gel, was coded RU.

The organism was studied in batch culture; maximal growth was reached after 48 hours, after which time the cells were in the 'stationary phase.' The endogenous respiratory activity of the cells rose to a maximum at 40 hours and then declined steadily, but the viable count remained fairly steady.

Analyses of the membrane fraction NM were made at various phases of growth up to a maximum of 160 hours. All the cell lipid was found to be concentrated in the membrane fraction. The amount of lipid expressed as a percentage of the salt-free dry weight of the cell remained constant, but both the total cell protein and the membrane protein fell during the period between 16 and 112 hours of growth. Also, the proportion of membrane to whole cell fell during this period. The menaquinone and the carotenoid pigment were localised exclusively in the membrane fraction. Both compounds exhibited maximal concentrations at 64 hours of cell growth and retained a constant molar ratio to each other regardless of the growth phase.

The crude (NM) and purified (R) membrane fractions were both affected by magnesium ion. In the absence of the ion, the membrane disaggregates to small lipoprotein particles. Magnesium ions also assist in the binding of the amino sugar layer of the cell envelope and of the P and RP fractions to the membrane.

The physico-chemical properties of the fractions NM, R, RP, RUP and P have been investigated by a combination of amino acid analysis, gel electrophoresis, sucrose density gradient centrifugation and gel filtration on Sephadex or Agarose. In addition, the binding of magnesium ion to the membrane and the isoionic point of the membrane fraction R have been determined.

The fraction R was found to be free from amino sugar and nucleotide. Fractions NM, R and RU contain all the cell lipid and cytochrome. Fractions NM and R, and probably also fraction RU, contain the cell menaquinone and carotenoid. Evidence is presented that suggests that the fraction R contains the NADH oxidase and adenosine triphosphatase of the electron transport system.

The action of the detergent sodium dodecyl sulphate (SDS) on the membrane fraction R was to break up the membrane into smaller particles. The disaggregation occurred in two distinct steps. The disaggregated particles could be reaggregated to a fraction which resembled the original membrane by removing the SDS by dialysis or gel filtration. The disaggregated particles and also the reaggregated membrane fraction were subjected to gel filtration, gel electrophoresis and sucrose density gradient centrifugation, in order to determine whether or not the lipid and protein components of the membrane had been separated.

A fraction which may be analogous to mitochondrial structural protein was isolated by ammonium sulphate fractionation of a preparation of fraction R dissolved in a mixture of sodium deoxycholate, sodium cholate and SDS.

CHAPTER I

INTRODUCTION

The term halophilic means salt-loving. Halophilic bacteria are organisms that have an essential requirement for sodium chloride for growth. Marine bacteria are halophilic, generally requiring about 2 to 5% sodium chloride as an essential component of their growth medium. Some bacteria are capable of surviving in media containing higher concentrations of salt and may even require such high concentrations. Bacteria which grow best in media of about 5 to 20% salt are termed moderate halophiles. Bacteria which grow best at between 20% and saturated (about 30%) salt are termed extreme halophiles. The extreme halophiles generally have a minimum requirement of about 10 to 15% salt for growth. Potassium ions, and to a lesser extent, magnesium ions, can partially replace the sodium ions (Brown & Gibbons, 1955; Christian, 1956; Weber, 1949) but no salts have yet been found that can completely replace the sodium chloride and yet maintain growth.

The extreme halophiles have a complex organic nutritional requirement. They do not generally metabolise carbohydrates, preferring proteins or amino acids as carbon sources. Chemically defined media (Dundas, Srinivasan & Halvorson, 1963; Onishi, McCance & Gibbons, 1965) have included at least ten amino acids and also nucleotides and glycerol. The extreme halophiles are usually cultured on protein digests (tryptone, peptone, yeast autolysate) containing suitable amounts of sodium chloride. If crude salt is used as the source of sodium chloride and if the medium is made up in tap water, the relatively small requirements for magnesium and potassium (Brown & Gibbons, 1955) and trace elements (Sehgal & Gibbons, 1960) will normally be satisfied.

The extreme halophiles are widely distributed in nature, mainly occurring in brines containing very high concentrations of salt. They are particularly conspicuous in the evaporation pans of salt works and in natural salt lakes of high salinity (e.g. the Great Salt Lake and the Dead Sea) where they often occur in such

large numbers that they impart a pink colour to the brine. Darwin had observed one such lake on his travels in 1833, and had attributed the red colouration to 'some infusorial animalcula' ("Voyage of H.M.S. Beagle," ch.IV, first published 1843). Many early references to the red colouration of salt and salterns, including a Chinese reference of c.2500 B.C. and a quotation from Pliny, are collected in Baas Becking (1931).

The extreme halophiles are also found in saline muds and soils throughout the world and are capable of survival in salt itself. Mined salt or rock salt is usually free from the organisms but solar salt, derived from the evaporation pans, frequently contains large numbers of the extreme halophiles (Larsen, 1962). Although storage of the salt under dry conditions tends to reduce the viable count, the bacteria have been successfully freeze-dried in the presence of salt medium. It is possible therefore that the organisms can remain viable in salt crystals when blown about by the wind, or otherwise transported across land.

The presence of extremely halophilic bacteria in crude solar salt is of considerable economic importance in many parts of the world. Salt is very often used as a preservative against microbial spoilage of food and other perishable goods, and the use of contaminated salt may well defy its own object. Visually, the bacteria reveal themselves as pink or red patches of slime; hence the "pink" or "pinkeye" of salted fish (Harrison & Kennedy, 1922; Finn, 1941; Shewan, 1942) and the "red heat" of salted hides (Lloyd, Marriot & Robertson, 1929; Lochhead, 1934). In severe cases the bacteria may completely penetrate the product. Such microbial spoilage is, of course, undesirable, particularly if the bacteria causes putrefaction as well as discolouration of the food product. However, in almost all cases investigated (Larsen, 1962) the cause of the contamination has been traced to the salt itself and spoilage may be avoided by the use of rock salt or sterilised sea salt.

However, it was the reddening of salted fish that first aroused interest in the extreme halophiles following the discovery that the discolouration was bacterial in origin. This was first realised by Farlow in 1878 (Farlow, 1880) and since then, a number of papers and reviews have been published on the general bacteriology of

the extreme halophiles. Many of these are listed in Larsen (1962), a good introductory review of halophilism.

Most, if not all, of the extreme halophiles are bacteria. Other halophilic organisms which have been reported have proved to be moderate rather than extreme. Thus, probably most of the species (approximately 27) isolated from the Great Salt Lake (Brock, 1969) are not optimally adapted to the high salt environment and only a few are extreme halophiles.

The main genus of extreme halophiles is Halobacterium. Bergey's manual (7th edition) lists five species, H. salinarium, H. cutirubrum, H. halobium, H. marismortui and H. trapanicum. In addition, two species of the family Micrococcaceae, Micrococcus morrhuae and Sarcina litoralis are extreme halophiles; these species may be variants of each other. Attempts to classify the various species of Halobacterium by their immunological response have been made recently by Zwilling, Rowen & Stotzky (1969), although H. salinarium, strain 1, was not included in the survey. Those strains which were tested fell into four mutually exclusive groups. A common genus-specific antigen and three group-specific antigens were demonstrated. The only other bacterial taxonomic group reported as having a representative which is an extreme halophile is the photosynthetic bacteria (Anderson, 1958; Raymond & Sistrom, 1967).

This thesis is primarily concerned with the halobacteria, in particular H. salinarium, strain 1. The halobacteria are obligate aerobes, and are usually red or pink. They appear red and transparent on slide cultures if non-vacuolated. Vacuolated strains are pink and opaque but appear red and transparent under pressure. The halobacteria are normally rod shaped, Gram-negative, non-spore forming and are lophotrichously flagellated if motile. H. salinarium, strain 1, is non-vacuolated and slightly motile. Cultures emit a characteristic odour which is partly attributable to hydrogen sulphide.

Cell morphology varies markedly with the concentration of salt in the medium. If the sodium chloride concentration of a suspension of halobacteria in concentrated salt solution is gradually lowered by the addition of water, the rod-shaped organisms are converted to spheres, via pleiomorphic, mostly club-

shaped intermediate stages, without any change in total cell volume. One rod-shaped organism normally gives rise to an average of about two spheres. On further diluting the suspension to about 10% (w/v) sodium chloride, the individual spheres suddenly lyse. It has been shown that some ions tend to protect the rod shape, e.g. K^+ , Mg^{++} , $SO_4^{=}$, $CH_3CO_2^-$, whilst others promote the deformation of the cells, e.g. NH_4^+ , Cd^{++} , CNS^- , ClO_4^- , $CCl_3CO_2^-$. The transformation and lysis processes do not appear to be enzymically mediated, although they are pH and temperature dependent.

The observation that the rod-sphere transition occurs without an apparent change in total cell volume (Mohr & Larsen, 1963) suggests that osmotic equilibrium is maintained throughout the diluting of the cell suspension. Cells grown in the presence of different concentrations of salt have an internal salt content which depends on, and approaches, the concentration of salt in the medium (Gibbons & Baxter, 1953; Holmes, 1964; Christian, 1956; Christian & Ingram, 1959). However, in addition to Na^+ and Cl^- , K^+ is a dominating component of the internal salt and its concentration approaches the solubility limit of KCl (Christian & Waltho, 1962). The concentration difference of Na^+ across the cell envelope is very approximately equal to that of K^+ but is of opposite sign.

The response of the extreme halophile to changes in the salt concentration of the medium is related to the instability of the cell envelope of the organism when exposed to hypotonic media (Larsen, 1967). Very similar structural transformations and lysis patterns are observed with isolated cell envelope preparations. However, K^+ is almost as effective as Na^+ in preserving intact cell envelopes and Mg^{++} is much more effective in maintaining the integrity of cell envelopes than it is in maintaining the rod-shape of whole cells.

The isolated enzymes of halobacteria also exhibit complex ion-effects. It is to Baxter & Gibbons (1954, 1956, 1957) that we owe the important discovery that the enzymes are themselves 'halophilic', i.e. are adapted to the high intracellular salt concentration. They mostly exhibit relatively high or even maximal activity in 25% (w/v) salt and are deactivated in the absence of salt. Two typical salt response curves are presented in Fig. 7.1. It may be significant that some

of the enzymes tested exhibit higher maximal activities in KCl than in NaCl solutions. The salt relations of a number of individual enzymes has been reviewed by Larsen (1967). A few reports concerning additional enzymes have appeared subsequently. Brown (1966) has reported an NADH oxidase and Stevenson & Brown (1967) an adenosine triphosphatase from H. salinarium, strain 1. Lanyi & Stevenson (1969) have reported a catalase from H. cutirubrum and Norberg & Hofsten (1969) have reported extracellular proteases from H. salinarium, strains 1, 1M and 5. All these enzymes exhibit one or other of the salt-response curves typical of halophilic enzymes (see Fig. 7.1.). However, the proteases are reported to be more active in KCl than in NaCl, unlike the majority of the other enzymes (see above). The salt relations of the enzymes are more fully discussed in Chapter VII.

The question of whether or not the halobacteria possess a cell wall is still debatable. For many years the halobacteria were thought to be almost unique in the microbial world in possessing only a single limiting plasma membrane for a cell envelope. In the electron microscope only the typical three-layered structure characteristic of the unit membrane (Robertson, 1966) was observed, although the structure was some 50 - 75% wider than the more usual 75 - 80 Å. More recently, however, the anatomy of the cell envelope of the halobacteria has been shown to be more complex (Larsen, 1967). With improved techniques of electron microscopy Stoeckenius & Rowen (1966, 1967) and Cho, Doy & Mercer (1967), working with H. halobium, and Steensland (1967a) and Steensland & Larsen (1969), working with H. salinarium, showed that the envelope consisted of a unit membrane overlaid by a proteinaceous coat 75 - 150 Å thick. Moreover, H. halobium contained a much more complex system of membranes than H. salinarium (Stoeckenius & Rowen, 1967; Stoeckenius & Kunau, 1968).

The envelope of H. salinarium, strain 1, has been further characterised by Steensland (1967b). The outer protein coat, which is responsible for the characteristic surface pattern of whole cells and isolated envelopes, is separated from the remaining envelope during exposure of the cells or envelopes to media of low salinity. The primary stabilising cation is sodium. If the envelope is exposed to a medium low in magnesium ion as well as sodium ion, an 'amino sugar layer' is

also released, as monitored by the release of amino sugar components from the membrane. Possibly this amino sugar layer corresponds to the mucopolysaccharide layer normally found in Gram-negative bacteria although other components characteristic of mucopolysaccharide appear to be absent. Muramic acid has not been found either by Brown & Shorey (1963) in H. salinarium and H. halobium or by Kushner, Bayley, Boring, Kates & Gibbons (1964) in H. cutirubrum. No diaminopimelic acid could be detected in cells of H. salinarium and H. halobium by Brown & Shorey (1963) and Steensland (1965) confirmed the absence of this component in H. salinarium. Kushner & Onishi (1968) have reported the absence of D-amino acids and techoic acid components in hydrolysates of the envelope of H. cutirubrum. Proliferating cells of H. salinarium are sensitive to penicillin but are unaffected by D-cycloserine (see Larsen, 1967).

However, the nature of the amino sugar components has been fairly well established by Mjelde (1967, 1968, 1969). A glucosamine residue is linked to a second unidentified amino sugar (not muramic acid) which is further linked to a peptide chain.

On dissolution of cell envelopes in water, both H. halobium and H. cutirubrum give only one or two major components as judged by the sedimentation pattern of the solutions in the analytical ultracentrifuge (Brown, 1963; Onishi & Kushner, 1966). At the time, it was generally thought that the cell envelopes of the halobacteria consisted only of the plasma membrane, so these authors naturally attributed the sedimentation pattern to lipoprotein subunits of the membrane. However, it is possible that the slowly sedimenting particles are, instead, subunits of the outer envelope, the membrane still existing as fragment, which sediment relatively quickly. Thus, Brown & Netschey (1967) observed a fast component that appeared during the acceleration of the centrifuge and accumulated on the bottom of the cell.

The major slowly sedimenting particle (Brown & Netschey, 1967) has an apparent molecular weight of 390,000, a partial specific volume of 0.790 ml./g. and an intrinsic viscosity of 0.600 dl./g. The value for the intrinsic viscosity is

high compared to the average globular protein (Yang, 1961) and may indicate that the particles are in a more extended form than in the native envelope in 4 M NaCl. In the electron microscope, the outer protein coat appears as a hexagonal array of subunits, approximately 130 Å in diameter (Larsen, 1967). An analysis of a fraction which probably corresponds to the outer layer in H. halobium has been presented by Marshall, Wicken & Brown (1969).

Only under carefully controlled conditions is a preparation of relatively pure plasma membrane obtained (Steensland, 1967b; Steensland & Larsen, 1969; Stoeckenius & Kunau, 1968). The preparative method involves primarily the exposure of the cell envelope to distilled water, and centrifuging the membrane fragments from contaminating envelope and cytoplasmic material. With H. halobium, (Stoeckenius & Kunau, 1968) a relatively complicated fractionation procedure must be employed to separate the various membraneous components (i.e. plasma membrane, intracytoplasmic membrane, gas vacuole, purple membrane). With H. salinarium, strain 1, the procedure is simplified by the presence of only the plasma membrane.

The choice of an appropriate biological system for an investigation into the structure and function of the cell membrane is a difficult one to make. The choice of the bacterial plasma membrane, specifically of H. salinarium, strain 1, was one that was based largely on the grounds of the ease of preparation of the membrane fraction. In general, the isolation of bacterial membranes is an easier problem than the isolation of membranes from animal cells, with the possible exception of the erythrocyte plasma membrane. However, the erythrocyte is the site only of limited biological activity. The bacterial plasma membrane-mesosome fraction contains many biochemical activities, in particular the electron transport system (Salton, 1964), the (associated) ion transport system (Kabach & Stadtman, 1966), and possibly is also the site of protein synthesis (Butler, Crathorn & Hunter, 1958), particularly of extracellular enzymes, (Bishop, 1964) and of the synthesis of cell wall lipopolysaccharide (Rothfield, Takeshita, Pearlman & Horne, 1966).

For most bacteria, the isolation of the plasma membrane-mesosome fraction is complicated by the presence of cell wall material external to the plasma membrane. For Gram-positive organisms, the cell wall can usually be cleanly removed by lysozyme treatment. Alternatively, the 'ready made' L-forms may be utilised prior to lysis of the cell in hypotonic solution. Gram-negative organisms, however, have a more complicated membrane structure (Murray, Steed & Elson, 1965; de Petris, 1965, 1967) consisting of an inner (cytoplasmic) and outer membrane on either side of the cell wall lipopolysaccharide/peptidoglycan. Only recently (Birdsell & Cota-Robles, 1967) has it been reported that the plasma and outer membranes have been isolated relatively free from the wall layer in Gram-negative organisms. The EDTA-lysozyme treatment involved also resulted in partial separation of the inner and outer membrane structures. With H. salinarium, which contains no apparent mesosome system and which possibly contains only a degenerate mucocomplex layer, the plasma membrane may be separated with comparative ease.

The other obvious candidates for a study of membrane structure are the species of Mycoplasma. Like the L-forms of Gram-positive organisms, they apparently contain little or no cell wall external to the plasma membrane. Mycoplasma have been fairly thoroughly investigated by Razin and his group (Razin, Argaman & Avigan, 1963; Razin, Morowitz & Terry, 1965) but little work has been done on the envelope structure of the halobacteria. Also, a representative of the extremely halophilic bacteria was chosen because the organisms are themselves of great interest on account of the unusual environment in which they live. As mentioned above, an interest in the halophilic nature of the cells leads directly to an investigation of envelope structure and hence also, to an investigation of the plasma membrane. Further, a specific salt requirement has been traced to an active transport system (Stevenson, 1966) and possibly also to oxidative phosphorylation (Brown, 1966). This again creates an interest in the plasma membrane - the site of these biochemical activities. If the membrane itself exists in a highly ionic environment, as might be supposed, polar forces will be shielded

and non-polar interactions will then become more important. Hence, the possibility arises that the non-polar interactions, which are becoming increasingly considered in current concepts of membrane structure, may be more amenable to study in halobacteria than in a conventional membrane.

CHAPTER II

METHODS

2.1. Organisms

Halobacterium salinarium (strain 1), Halobacterium salinarium (strain 1M, a colourless mutant of strain 1) and Halobacterium cutirubrum were gifts from Professor Helge Larsen, Institutt for Teknisk Biokjemi, Norges Tekniske Høgskole, Trondheim, Norway.

2.2. Cultivation

The organisms can be maintained lyophilised for long periods. Routinely they were preserved in an actively growing state by regular transfers from a 'shake' culture.

For the work concerned with variations in composition of the membrane of H. salinarium with the age and nutritional status of the organism, ('ageing studies'), the liquid culture medium had the following composition (% w/v): 25 crude solar salt, 0.5 KCl, 0.5 NH_4Cl , 0.5 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 5% (v/v) yeast autolysate nutrient, pH 7.0, which contained 20% (w/v) Difco yeast extract and 10% (w/v) Oxoid tryptone. The salts solution and nutrient were made up in tap water and were autoclaved separately. When cells were needed for analysis, an enriched culture was prepared; the normal inoculum of 5% (v/v) was increased to 20% (v/v) and the nutrient in the medium was increased to 10% (v/v). For both routine and enriched cultures, 50 ml. volumes were incubated at 37° in 250 ml. conical flasks in a Gallenkamp orbital incubator at 250 rev./min. Two enriched cultures, three days' old, were then used to inoculate 500 ml. volumes of enriched culture medium. These were then incubated at 37° in 5l. round bottomed flasks set at 45° to the horizontal and rotated at 200 rev./min.

For subsequent experiments, described in chapters IV to VII, a slightly different culturing procedure was employed. The yeast autolysate nutrient in the culture medium was replaced by 1% (w/v) Oxoid bacteriological peptone. The

intermediate, 50 ml. enriched culture stage was omitted, routine shake cultures being used to inoculate the 51. round bottomed flasks, in which the volume of (unenriched) culture medium was increased to 1 l.

2.3. Harvesting of cells

The large cultures, 560 or 1100 ml. each, were harvested after 1 to 5 days' growth ('ageing studies') or at the end of the exponential phase, 66 hr. of growth (other experiments), respectively. The cultures were cooled rapidly to -10° , sedimented and washed in a Sorvall RC2-B centrifuge at 4000 g (10 min.) using rotors GS-3 or GSA, depending on the volume to be harvested. Usually three cultures were handled at a time.

2.4. Preparation of cell envelope fragment bags (CEFB)

An equal volume of glass beads (Glasperlen, 0.17 - 0.18 mm. diameter, B. Braun, Melsungen) was added to a pellet of washed cells and the whole was suspended in Analar salts medium of composition (% w/v): 25 NaCl, 0.5 KCl, 0.5 NH_4Cl and 0.5 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, containing 0.1 M tris. HCl, pH 8.0. The buffered Analar salts medium is designated buffer α . The suspension was homogenised for 2 min. at full speed in a Braun homogeniser, pre-cooled with liquid CO_2 . Glass beads and cell debris were sedimented at 4000 g (10 min.) using the Sorvall GSA rotor. The red supernatant was then centrifuged at 27,000 g (1 hr.) to yield a red pellet. The supernatant was discarded and the pellet was resuspended in buffer α and re-centrifuged, first at 4000 g (pellet discarded) and then at 27,000 g (supernatant discarded).

2.5. Preparation of crude membranes (NM)

A pellet of washed cells ('ageing studies') or a pellet of CEFB (other experiments) was resuspended in buffer α (5 volumes) and was dialysed against 1250 volumes of distilled water (14 hr.) and then against a further 1250 volumes of distilled water (1 hr.). All dialyses were performed at 2° . The suspension was then treated with nucleases, as suggested by Steensland & Larsen (private communication) but with certain minor modifications. The suspension was

adjusted to 0.1 mM tris. HCl, pH 8.0 and 10 mM MgCl_2 . 30 μg each of deoxy-ribonuclease and ribonuclease/mg. protein were added, and the suspension was warmed to room temperature and stirred gently for 1 hr. The nuclease-treated suspension was then diluted with 4 volumes of ice-cold distilled water, and the membrane-rich fraction was sedimented by centrifuging at 150,000 g (4 hr.) in a Beckman Spinco L-2 ultracentrifuge at 0° using rotor 50. The pellet was washed by centrifuging twice from 2 mM MgCl_2 . The final washed pellet was designated fraction NM.

2.6. Salt-free dry weights

Membrane preparations were dialysed exhaustively against distilled water and dried to constant weight at 105° . Whole cell preparations were dried to constant weight at 105° and the weight of the residue, obtained by ashing at 400° , subtracted.

2.7. Protein determination

Protein was estimated by the Folin-Ciocalteu method (Lowry, Rosebrough, Farr & Randall, 1951) using bovine serum albumin as a standard. Whole cells were solubilised with sodium deoxycholate, pH 12. Samples estimated after trichloroacetic acid precipitation gave essentially the same results, except that the Folin-Ciocalteu estimation gave a high blank and was of low sensitivity for samples in 6 M urea. These preparations were therefore estimated preferentially by precipitating the protein with trichloroacetic acid and dissolving the precipitate in sodium deoxycholate, pH 12, before the estimation.

2.8. Nucleic acid determination

Nucleic acid and nucleotide were estimated by the orcinol reaction (Schneider, 1957).

2.9. Cytochrome estimation

Cytochromes were estimated by difference spectra, using sodium dithionite as reducing agent. For convenience, cytochrome content was expressed in arbitrary units (a.u.) defined as ΔE at 427 nm - ΔE at 480 nm on reduction

(Bednar, 1965). For concentrated membrane samples, a Unicam SP500 or Zeiss PMQ II was used for the spectral analysis (wavelengths 2 nm higher on Zeiss). Whole cell preparations were solubilised by diluting with 10 volumes of distilled water and treating with nucleases. A Beckman DK-2B spectrophotometer was used for the spectral analysis.

For the analysis of minor cytochrome components, preparations from a colourless mutant of H. salinarium, strain 1M were used. The absence of carotenoid (light-absorption max. in water 500 nm) facilitated cytochrome estimation. Membranes (NM) were prepared from cells of the mutant, omitting the CEFB stage, and ascorbate-N,N,N',N'-tetramethyl-p-phenylenediamine was used as the reducing agent. The Beckman instrument was used for the spectral analysis.

2.10. Amino acid analysis - hydrolysis

The protein or lipoprotein samples were dialysed against at least eight changes of distilled water over a period of 3 days at 2°. Duplicate aliquots containing approximately 1 mg. protein were pipetted into heavy walled Pyrex digestion tubes (18 x 120 mm. with a 100 mm. stem of 6 mm. inner diameter) and were frozen in liquid nitrogen and lyophilised. 0.25 ml. micro-analytical grade concentrated HCl (freshly opened bottle) and 0.25 ml. 0.001 M norleucine in 0.01 M HCl were added and the tubes were immersed in liquid nitrogen until the contents were just frozen. The tubes were then sealed under vacuum (< 50 microns) allowing the contents to thaw in order to remove traces of dissolved air.

The tubes were then maintained at $110^{\circ} \pm 1^{\circ}$ for 22 hr. in a heating block in an air oven provided with forced air circulation. After cooling to room temperature, the tubes were opened and the HCl removed under vacuum.

HCl was normally removed after hydrolysis by placing the tubes in a desiccator over KOH in preference to the use of a rotary evaporator, since the hydrolysate volume was small (Beckman Amino Acid Analyser Manual, A-1M-3, 1965). However, it has been reported (Moore & Stein, 1963) that

this procedure may lead to decreased yields of serine and threonine and the appearance of several small new peaks on the chromatograms.

Ikawa & Snell (1961) have suggested that the formation of glutamyl serine is also greater during the evaporation if vacuum desiccation is employed instead of rotary evaporation.

No chromatographic evidence was found for any of these artefacts in accordance with Crestfield, Moore & Stein (1963) who found only barely detectable quantities after 24 hr. in a desiccator.

When the hydrolysate was dry (normally after 4 - 12 hr. in the desiccator) the film was dissolved in 5 ml. of freshly prepared pH 2.2 buffer (Moore & Stein, 1954) and stored at 0° until analysed.

2.11. Amino acid analyser - equipment changes

The analysis of protein or lipoprotein hydrolysates was performed on a Beckman model 120C amino acid analyser set up as described in the Instruction Manual (A-1M-3, 1965) with the following modifications :

The AA 27 resin supplied for the analysis of basic amino acids gave a poor resolution of tryptophan and lysine. It was therefore replaced by the PA 35 resin which was originally supplied for physiological fluids analysis and which gave a better resolution per unit of column length than the AA 27 resin. In addition, the length of the resin column was increased from 5 cm. to 7 cm.

Occasionally very high back pressures were built up in the 7 cm. column due to compression of the resin, which tended to increase throughout the duration of each analysis. The high operating pressures could be relieved by presoaking the chromatography column in 50% NaOH overnight, which presumably etched the column and minimised movement of the resin in the column when under pressure.

To increase the sensitivity of the apparatus for the detection of sub-micro quantities of amino acids, an additional flow cell was included in the photometer assembly. The additional flow cell was modelled on the horizontal flow cell described by Jones & Weiss (1965). The flow cell was machined from a block of teflon, 25 x 38 mm. cross section, 16 mm. long, the flow path being 3 mm. in

diameter and 10 mm. long. Windows and tubing connections were fitted as described by Jones & Weiss (1965). Instead of fitting a slide holder to the cuvette mounting plate, the additional flow cell and the alternate 2.2 mm. flow cell were mounted together between brass plates and the whole assembly was inserted in a groove cut in the mounting plate. The slide assembly could be shifted reproducibly between two positions governed by end stops. Both cells when positioned in the light path were at the original optical centre of the colorimeter, so that no modifications of the light source were necessary. The long path length cell gave an amplification of peak area, compared to the 2.2 mm. cell, of 4.60. If the amplification were strictly linear with path length, the factor would be 4.55.

2.12. Analysis of protein hydrolysates

The analysis of protein hydrolysates was performed as described in the Instruction Manual (A-1M-3, 1965) supplied with the Beckman model 120C amino acid analyser used. The short column analysis time was increased slightly due to the lengthened resin column (see 'Equipment changes'). A modified long column analysis was employed for amino sugar determinations (see 'Amino sugar analysis'). In addition to the standard protein hydrolysate amino acids (Beckman "calibration mix") the following amino acids were observed on chromatograms :

a) Cysteine, cystine and cysteic acid

The cystine peak obtained when a hydrolysate was analysed was not symmetrical ; racemisation of cystine during hydrolysis gave rise to meso-cystine as well as the D, L-racemic mixture. The meso-form was almost completely separated from the racemic mixture and was positioned 5.5 min. ahead of the racemic mixture. Cysteine, when present, was positioned 2 min. ahead of proline but could be easily recognised by comparing the ratio of the readings obtained at 440nm. and 570nm. Cysteic acid, when present, was positioned 30 min. before aspartic acid.

b) Tryptophan

Tryptophan, when present, was well resolved from lysine, being positioned

3 min. ahead.

c) Methionine sulfoxides

Methionine sulfoxides, which emerged just ahead of aspartic acid, were assumed to be derived from oxidation of methionine, and methionine values quoted include methionine sulfoxides.

d) Methionine sulphone

Methionine sulphone was positioned 3.5 min. ahead of threonine.

e) Allo-isoleucine

Allo-isoleucine, which emerged 3.5 min. ahead of isoleucine was assumed to be derived from isoleucine, and isoleucine values quoted include allo-isoleucine.

f) Norleucine

Norleucine, used as an internal standard, was positioned 3.5 min. after leucine.

g) Ethanolamine

Ethanolamine was positioned 1.5 min. after ammonia; two other peaks on the short column, one 1.9 min. after lysine and one 4.2 min. after arginine, were not identified.

Corrections

Correction has been made for expected losses of serine (10%), threonine (5%), tyrosine (5%) and glutamic acid (5%) as estimated by Hirs, Moore & Stein (1956). Corrections have been made to the value obtained for ammonia by

- i) subtracting moles of ammonia equivalent to the expected losses of serine and threonine (above) and
- ii) subtracting moles of ammonia that were expected to have been absorbed by the sample from the atmosphere during the hydrolysis and chromatography (computed from a blank run).

Contributions from the hydrolysis of lipid present in the lipoprotein samples was expected to be low, since the lipid is very low in nitrogen (Steensland & Larsen, 1969). Nevertheless, ammonia values quoted are probably, in most cases, too high, particularly in the case of samples exposed to urea.

Values for tryptophan and cysteine (sum of cysteine, cystine and cysteic acid) are also included, but these are probably minimum values. No attempt was made to estimate these amino acids by more reliable methods, except for tryptophan which was estimated in R fraction spectrophotometrically by the method of Beaven & Holiday (1952).

The amino acid compositions of fractions are tabulated as anhydro amino acid/100 mg. salt free dry weight and as mole %, excluding ammonia. The recovery of amino acids through the hydrolysis procedure and chromatography was estimated by the use of norleucine as an internal standard (Walsh & Brown, 1962). Recovery of norleucine was on average 93%.

2.13. Amino sugar analysis

Fractions for analysis were concentrated by extracting the lipid and the majority of the protein by using the lipid extraction procedure of Bligh & Dyer (1959), c.f. section 2.14. The lower chloroform phase, containing the lipid, was discarded together with the layer of denatured protein which collected at the interface of the two phases on centrifugation. The upper methanol-water phase was reduced to small bulk in a rotary evaporator at 40° and excess salt filtered off. Aliquots were then hydrolysed for 4 hr. at 105° in 4 M HCl in sealed tubes under vacuum. After cooling, the tubes were opened and the HCl evaporated under reduced pressure. Aliquots of the residue were dissolved in pH 2.2 buffer (Moore & Stein, 1954) and stored at 0° until analysed.

Samples were then processed as for a normal amino acid analysis. Amino sugar (as glucosamine) could be detected on the short column, between the 'acids and neutrals' peak and tryptophan. However, the various amino sugars, e.g. glucosamine and galactosamine, could not be resolved adequately on the short column. A modified elution programme on the long column (69 cm.) enabled

glucosamine and galactosamine, and also muramic acid, diaminopimelic acid and norleucine to be resolved from the standard protein hydrolysate amino acids. The pH 3.28 to 4.28 buffer change was set at 137 min. and the shut-down timer at 250 min.

2.14. Extraction of lipids

Total lipids were extracted from preparations by the method of Bligh & Dyer (1959) except that the filtration step was replaced by centrifuging in glass bottles. The Sorvall GSA rotor was used in the RC2-B centrifuge; spins were for 10 min. at 2000 g. After centrifuging, a buff, sticky layer of denatured protein and nucleic acid collected at the interface of the methanol and chloroform layers. This was re-extracted by repeating the original extraction. Combined chloroform layers were then reduced to small volume by distillation and taken to dryness under nitrogen. The residue was re-extracted with dry chloroform to constant weight. The extraction procedure and all subsequent manipulations were carried out in subdued light. Lipid samples were stored at -20° under vacuum in the presence of a self-indicating dessicant (silica gel). Non-saponifiable lipid was extracted by the method of Kates, Palameta, Joo, Kushner & Gibbons (1966).

2.15. Lipid analyses

Total lipids were estimated gravimetrically. Lipid phosphorus was estimated by the method of Rhee & Dugan (1967) using sulphuric acid - 70% (w/v) perchloric acid (1 : 1 v/v) as digesting reagent and 1-amino-2-naphthol-4-sulphonic acid as reducing agent. Similar results were obtained by the method of McClare (private communication) using 70% (w/v) perchloric acid as digesting reagent and ascorbic acid as reducing agent. Vicinal glycols were determined by periodate oxidation (Karnovsky & Brumm, 1955) followed by a formaldehyde determination with chromotropic acid (MacFadyen, 1945).

The general method employed for the analytical fractionation of the complex lipid extract obtained from H. salinarium was a combination of DEAE-cellulose column chromatography and thin-layer chromatography (Rouser, Galli, Lieber, Blank & Privett, 1964). The DEAE-cellulose column fractionation was designed

to separate major lipid classes, this method being used in preference to methods of separation based on the solubility properties of lipid classes, as recommended by Lees (1957). The precipitation of phospholipids from chloroform solution by acetone (Fauré, Maréchal & Troestler, 1964) had, in any case, not been successful.

2.16. Column chromatography of lipids - DEAE-cellulose

The method used was essentially that of Rouser, Kritchevsky, Galli & Heller (1965). DEAE-cellulose (5 g. Whatman DE-50) was pre-washed with three cycles of 1 M HCl and 0.1 M KOH, washed with water (3 vol.), acetic acid (3 vol.) and methanol (3 vol.) and then air dried and desiccated over KOH. The resin was converted to the acetate form by standing it overnight in acetic acid. The resin was packed in a column of 1 cm. inner diameter, and washed with methanol (4 vol.) and chloroform (4 vol.). The mixture to be analysed (100 mg.) was applied to the top of the column as a chloroform solution. Non-polar lipids were eluted from the column with chloroform, polar lipids with methanol-chloroform (1:2, v/v) and chloroform-methanol-28% (w/v) aqueous ammonia (8:4:1, by vol.). After use, the resin was regenerated by washing with methanol and chloroform.

2.17. Column chromatography of lipids - Decalso

Decalso (5 g. Permutit) was packed in a column of 1 cm. diameter, fitted with a tap lubricated with charcoal, as a slurry in light petroleum (40 - 60°). The mixture to be analysed, the non-polar lipids from the DEAE-cellulose fractionation (see section 2.16), 10 mg., was applied to the top of the column as a suspension in light petroleum, after pre-washing the column with light petroleum. Non-polar lipids were eluted with light petroleum (100 ml.). A fraction containing menaquinone was eluted with 2% (v/v) of diethyl ether in light petroleum (75 ml.) and finally most of the remaining lipid was eluted with methylal (dimethoxymethane).

2.18. Column chromatography of lipids - Alumina

Acid-washed alumina (5 g. Woelm, Grade II) was packed in a column of 1.6 cm. diameter as a slurry in light petroleum (40 - 60°). The lipid fraction to be analysed, 50 mg. non-saponifiable lipid (see section 2.14.), was applied to the top of the column as a slurry in light petroleum. Lipid fractions were eluted

in light petroleum followed by increasing concentrations (v/v, 2%, 4%, 6%, 8%, etc.) of diethyl ether in light petroleum.

2.19. Thin-layer chromatography

250 μ m thick plates, 20 cm. x 20 cm., of silica gel H (Merck) were prepared and scored as in the "TLC path method" of Blank, Schmidt & Privett (1964) to improve resolution. Plates were activated by drying at 110° for 1 hr. After the application of the lipid samples, plates were developed in various solvent systems in conventional tanks lined with filter paper. For the analysis of total lipids or of phospholipids, solvent system A was used, which was chloroform-methanol-water (65:25:4, by vol.). For the analysis of neutral lipid fractions, either solvent system B, diethyl ether-light petroleum (40 - 60°)-acetic acid (30:170:1, by vol.) or solvent system C, methanol-chloroform (1:4, v/v) were used.

Lipids were visualised by charring with 50% (v/v) H_2SO_4 or by spraying with Rhodamine 6G (Marinetti, Erbland & Kochen, 1957). Phospholipids were detected with the Hanes-Isherwood spray (Hanes & Isherwood, 1949). Cis-glycols were detected by the periodate-Schiff reaction (Sastry & Kates, 1964).

2.20. Estimation of Menaquinone and carotenoid

Menaquinone was estimated directly from the fraction of non-polar lipid (see section 2.16.) eluted from the Decalso column in 2% diethyl ether in light petroleum (see section 2.17.) from the u.v. absorption at 249 nm. in cyclohexane solution using an $E_{1\text{ cm}}^{1\%}$ value of 264 (Bishop, Pandya & King, 1962). A gravimetric estimation of this fraction indicated that it was 90 - 100% menaquinone (estimated spectrophotometrically).

Carotenoids were extracted from preparations suspended in 15% (w/v) NaCl solution with 2 vol. acetone, and transferred to ether. An $E_{1\text{ cm}}^{1\%}$ value of 2500 at 494 nm. was used (Liaaen Jensen, 1962). Spectrophotometric data were obtained on a Zeiss PMQ spectrophotometer.

2.21. Gel filtration

For the routine purification of crude membrane preparations, a large column, 45 cm. x 3.6 cm. inner diameter, of Bio-Gel A-50m agarose (Bio-Rad)

was used. For molecular weight estimations and for various fractionation procedures using detergent or urea, smaller diameter columns were used; 50 - 100 cm. x 1.6 cm. inner diameter, using Bio-Gel A-150m or A-50m, or Sephadex G-200, G-100 or G-25 (Pharmacia) as gel filtration resin.

In most experiments, a basic buffer, designated buffer β , of 0.1 M NaCl, 0.05 M tris. HCl, pH 8.0, and 0.02% sodium azide (as a bacteriostat), was used. In some experiments 1 - 10 mM MgCl_2 , 0.4 - 10 mM sodium dodecyl sulphate (SDS) or 6 M urea were added to the basic buffer.

Columns were run under reversed flow, as this minimised column packing and resultant high back pressures. Buffer was pumped upwards through the column by means of a peristaltic pump (LKB 4917A) and the sample was applied by temporarily removing the tubing connection from the buffer reservoir and placing it in the sample tube. By this procedure, it was rendered unnecessary to stop the flow of liquid through the column, which tended to cause disturbances in the Uvicord. The small bubbles introduced were removed by the use of a bubble trap. Effluent fractions were monitored on an LKB Uvicord (type 4710A) and collected on a fraction collector (LKB 3404B) operated on a timed auto change.

Samples applied were approximately 20 mg. protein per cm. cross section of resin and flow rates were approximately 18 ml./hr. except for the columns run in buffer containing 6 M urea, when the flow rates were 10 ml./hr. Blue dextran 2000 (Pharmacia), aldolase, hexokinase, haemoglobin and ribonuclease A (Sigma) and digitonin (Calbiochem) were used as molecular weight standards, on columns equilibrated with buffer β . All molecular weights obtained are calculated on the assumption that the calibrations with columns equilibrated with buffer β are appropriate when buffers containing urea or detergent were used. There is some evidence that this assumption may not be valid; Rosenberg & Guidotti (1967) have reported that the pore size of some gel filtration resins are altered in the presence of unbuffered SDS. No attempt was made to confirm this or otherwise for it was expected that the calibration proteins would also be susceptible to, for example, binding by SDS, but possibly to different extents, so that they would cease to be standards. It must therefore be remembered that all such molecular weights

quoted are 'apparent.' In the case of samples in detergent, the apparent molecular weights may be adjusted to account for the (known) bound SDS.

2.22. Polyacrylamide gel disc electrophoresis

All the electrophoretic experiments were run by Mr. F. Bellingham (with the exception of some abortive experiments with cellulose acetate membranes) but are included in this thesis since they provide valuable supplementary data. Relevant details of the methods used are as follows :

Samples, 200 μ g. of protein, were layered onto a small-pore gel (7%) overlaid with a large-pore spacer gel (3.5% acrylamide). The buffer used was 0.03 M sodium borate, pH 8.0, and the lower electrode was the anode. After developing, the gels were fixed in trichloroacetic acid and stained for protein (Coomassie blue), lipid (Oil red O) or nucleic acid (methylene blue).

Some of the polyacrylamide gel disc electrophoresis analytical runs were scaled up and run on a preparative electrophoresis apparatus. The electrophoresis column was eluted near the base of the gel by passing a stream of buffer β , without NaCl, through a thin section cut through the gel, whilst the run was in progress. Fractions were collected and monitored as for the gel filtration experiments.

2.23. Isoionic point determination

Purified membrane-rich fraction (R) in 0.1 M NaCl, 0.05 M tris. HCl, pH 8.0, was concentrated by ultrafiltration (Amicon Diaflo, filter UM2) to 10 - 20 mg. protein/ml. The sample was then desalted by passage through a column of mixed ion exchange resins as described by Nozaki and Tanford (1967). The solution of the desalted lipoprotein obtained from the ion-exchange column had a pH which varied with its protein concentration, as measured by its absorption at 280nm. A plot of the pH of the desalted lipoprotein solution against the reciprocal of its protein concentration was linear over the region $E_{280\text{nm}} = 0.2$ to 2.0 and a reproducible value could be obtained for the isoionic point of the lipoprotein by extrapolation of this plot to infinite protein concentration. Unusually low values for the isoionic point (a shift of up to 1 pH unit) were obtained for concentrated solutions of desalted lipoprotein ($E_{280} > 2.0$) where precipitation had occurred.

2.24. The binding of magnesium ion to the membrane-rich fraction (R)

R fraction, 20 mg. protein, was dialysed exhaustively against 1 mM EDTA pH 7.0 and then deionised water. Magnesium binding was determined by the equilibrium dialysis method. The lipoprotein was placed in a washed dialysis bag, vol. 30 ml., and immersed in deionised water, vol. 170 ml., to which known quantities of magnesium, as $MgCl_2$, were added. Both the internal and the external solutions were stirred for 24 hr. at 2° . After equilibration, magnesium in the external medium was determined by atomic absorption spectrophotometry. It was assumed that the lipoprotein was isoionic and that the net charge on the lipoprotein was negligible (Steinhardt & Beychok, 1964). Bound magnesium was calculated by the method of Scatchard, Scheinberg & Armstrong (1950) assuming no chloride ion was bound. Blank measurements, without magnesium chloride and without lipoprotein, were also made.

2.25. Endogenous activity of whole cells

Cells were harvested as described above and washed several times with buffer α until free of growth medium. For each wash, a cell pellet was re-suspended with extreme care in buffer α ; the pellet first being 'softened' by exposure to buffer for 30 min. and then resuspended in 50 volumes of buffer using a pipette with a wide orifice (1 mm.). Cells were maintained at 0° at all times except during centrifuging, when they were maintained at -10° . For the investigation of the effect of the age of the culture on the endogenous activity of the cells, cultures were grown such that they could all be assayed on the same day.

A conventional Clark type of oxygen electrode was used to measure endogenous oxygen uptake polarographically. The cathode was maintained at -0.6 v and the generated current measured with a Kent mark 3 (0 - 1 mv) or Servoscribe (0 - 2 mv) recorder. The endogenous activity of cells, suspended in buffer α was assayed in buffer α at 30° . Due allowance was made for the limited solubility of oxygen in concentrated salt solutions, an estimate of the solubility of oxygen in 4.3 M NaCl being interpolated from the results of

Smith (1928).

2.26. Assay of NADH oxidase

The NADH oxidase activity of whole cells and of envelope fractions was measured in two ways. In some experiments oxygen uptake was measured, as for the endogenous activity of whole cells, with an oxygen electrode. In some experiments, NADH oxidation was followed spectrophotometrically. A Zeiss M4 QII monochromator was linked to a Gilford 2000 recording spectrophotometer and the cuvette assembly was maintained at 30° . NADH oxidation was followed at 340nm (NADH, $\epsilon = 6220$, Horecker & Korinberg, 1948; NAD^{+} , $\epsilon = 20$, Siegel, Montgomery & Bock, 1959). Approximately 1 mg. protein of enzyme was used per assay. Activities were measured at the optimal NADH concentration (0.04 mM) for the NADH oxidase of an envelope fraction (CEFB) obtained by Brown (1966).

2.27. Assay of NADH-ferricyanide oxidoreductase ("diaphorase")

An enzyme that catalysed the transfer of electrons from NADH to ferricyanide (as the potassium salt) was assayed spectrophotometrically on the Gilford-Zeiss assembly as was used for the NADH oxidase assay (section 2.26.). NADH oxidation was followed at 340nm, and ferricyanide reduction at 420nm. (ferricyanide and ferrocyanide extinction coefficients obtained from Cohen & Plane, 1957). The assays were performed at substrate concentrations of $21 \mu\text{M}$ NADH and $50 \mu\text{M}$ $\text{K}_3\text{Fe}(\text{CN})_6$ in buffer α at 30° . Due allowance was made for residual NADH oxidase activity and for the non-enzymic reduction of ferricyanide by NADH, which were both small compared with the diaphorase activity. As the reaction was stoichiometric (1 mole of NADH reduced 2 mole $\text{K}_3\text{Fe}(\text{CN})_6$), the reaction was normally monitored by NADH oxidation only.

In order to enhance the observed activity (sometimes attributed to a "diaphorase"-type enzyme), preparations were, in some cases, treated with chaotropic agents (Davis & Hatefi, unpublished communication). As will be seen below (section 7.4.) chaotropic agents, especially thiocyanate (SCN^{-}) and perchlorate (ClO_4^{-}), release an NADH-ferricyanide oxidoreductase ("ferricyanide oxidoreductase") of activity up to one hundred times the diaphorase activity. In these experiments, buffer α was replaced by 0.1 M tris. HCl, pH 8.0, 100 mM Mg^{++} .

2.28. Assay of ferricyanide reductase activity

This activity is defined as the NADH-ferricyanide oxidoreductase activity released by chaotropic agents from preparations of CEFB. The effectiveness of various chaotropic agents in releasing this activity was investigated. The trial assays were as for the diaphorase assay, but chaotropic agents were included in the assay medium at concentrations up to 4 M. Unless otherwise stated in the results, the enzyme was added to the assay medium last, but in some cases the enzyme was pre-incubated with NADH or with the chaotropic agent.

As a result of these trial assays, the results of which are presented in section 7.5., a standard assay for the ferricyanide reductase was devised in which the chaotropic agent was perchlorate at a concentration of 3.3 M. Although thiocyanate was more efficient, mole for mole, than perchlorate in releasing the ferricyanide reductase activity, perchlorate was initially used in preference because it could be obtained in a higher state of purity. In some later experiments, when the potentially explosive nature of perchlorate was realised, thiocyanate was used in preference. In addition, when the stimulatory effect of imidazole had been observed, the tris buffer in the assay medium was replaced by imidazole at the same molarity and pH.

2.29. Incubation of cell envelope fragment bags with hypotonic media

Concentrated suspensions of CEFB (100 mg. protein/ml.) in buffer α were exposed to hypotonic media at 0°. The diluting buffer consisted of 0.1 M tris. HCl, pH 8.0 containing up to 100 mM MgCl₂ and up to 33 mM spermine; N,N'-bis(3-aminopropyl) 1,4-butanediamine. Normally the dilution was 1:50 (v/v), i.e. the salt was diluted to 86 mM, but in some experiments higher dilutions were obtained by centrifuging (150,000 g. for 2 - 4 hr. in the Spinco ultracentrifuge, rotor 50) and resuspending several times in the diluting buffer. The diluted preparations, or the final pellet and the various washings, were assayed for the chaotropically released ferricyanide reductase as described in section 2.28.

In a further set of experiments, preparations of CEFB that had been exposed to, and washed in, hypotonic media were exposed to a medium containing 4 - 6 M

urea in 0.1 M tris. HCl, pH 8.0, and again assayed for ferricyanide reductase.

In some experiments the effect of substituting 0.1 M imidazole buffer for the tris buffer in the above incubation was studied. For these experiments the revised thiocyanate-imidazole assay was used for the ferricyanide reductase activity (see section 2.27.).

2.30. Spectra of lipid samples

I.r. spectra of lipid samples were taken on thin films deposited from chloroform or light petroleum (40 - 60°) solution onto NaCl plates. Samples were desiccated for 1 hr. before spectra were taken. The spectrophotometers used were the Perkin-Elmer 137 (linear wavelength scale) and the Unicam SP-200 (linear wavenumber scale).

U.v. spectra were taken in cyclohexane, ethanol or other suitable solvent on the Unicam SP-800.

2.31. Chemicals

Unless otherwise stated, all chemicals were obtained from Hopkin and Williams or British Drug Houses Ltd. ; biochemicals were obtained from the Sigma Chemical Company. Wherever possible, AnalaR grade reagents were used, except for the growth medium for cell culture.

CHAPTER III

RESULTS I

VARIATION IN THE COMPOSITION OF THE PURIFIED MEMBRANE FRACTION (NM) WITH THE PHASE OF GROWTH

3.1. Introduction

It is well known that variations in cell composition and the activity of cell enzymes occur during the growth cycle of bacteria (Neidhardt, 1963 ; Glaser, 1966). Variations may occur during the logarithmic phase ('growth') as in Aerobacter aerogenes (Dean & Hinshelwood, 1959) or during the stationary phase ('ageing') as in Escherichia coli (Clifton, 1966) and/or in the transition between the logarithmic and stationary phases, as in Haemophilus parainfluenzae (White, 1962) or in Bacillus subtilis (Coleman, 1967). There is also evidence that (average) cell size may change during the growth and ageing of a culture (Dean & Hinshelwood, 1959 ; Salle, 1967).

It was of interest to see whether these overall changes during growth and ageing might be reflected in changes at the membrane level. At the time that this investigation was begun, no data were available concerning changes in the gross composition of membranes derived from Gram-negative organisms. Changes in membrane levels and composition during the phase of growth had, however, been noted for a number of Gram-positive organisms, from which protoplasts (plus mesosomes) could be readily obtained (Salton & Freer, 1965 in Micrococcus lysodeikticus and Sarcina lutea ; Shockman, Kolb, Bakay, Conover & Toennies, 1963 in Streptococcus faecalis).

For Gram-negative organisms only somewhat indirect evidence was available. Changes in respiratory activity or cytochrome levels, presumably changes at the membrane level, had been noted for a number of organisms (Englesberg, Gibor & Levy, 1954 in Pasteurella pestis ; Moss, 1956 in Aerobacter aerogenes ; White, 1962 in Haemophilus parainfluenzae), but

these changes were primarily a response to changes in oxygen tension as all three organisms are facultative anaerobes (Wimpenny, 1969).

With this background the aim of the present investigation was to determine the variations, if any, in membrane composition during the growth cycle of H. salinarium. The cells were grown under conditions where the lag phase was of minimal duration (use of ammonium chloride, 0.5%, w/v, in the growth medium, Onishi, McCance & Gibbons, 1965; use of a high inoculum, Holmes, Dundas & Halvorson, 1965), and cultures were harvested and examined at intervals throughout the logarithmic phase and from early to middle stationary phase (Fig. 3.1.). The gross composition of the membrane (preparation NM) was studied, both protein to lipid ratios and membrane to whole cell dry weight ratios, and the composition of the membrane lipid was investigated further, as a function of cell age. In the absence of a previous analysis of membrane or whole cell lipids of H. salinarium, a 'fingerprinting' technique was used to monitor the levels of the major lipid components, similar to that used by Dawson (1966) for the analysis of amino acid pools during growth in Candida utilis. Nevertheless, with the aid of partial analyses of whole cell lipids of other species of Halobacterium (H. cutirubrum; Sehgal, Kates & Gibbons, 1962; Kates, Sastry & Yengoyan, 1963; Kushner, Bayley, Boring, Kates & Gibbons, 1964; Kates, Yengoyan & Sastry, 1965; Fauré, Maréchal & Troestler, 1963, 1964) partial characterisation of the membrane lipids of H. salinarium was achieved. It should be added that a later report (Kates, Palameta, Joo, Kushner & Gibbons, 1966) had suggested that the cell lipids of a strain of H. salinarium were similar to those of H. cutirubrum, and also that it had been reported that the cell envelope, and therefore possibly the membrane, of H. cutirubrum (Smithies, Gibbons & Bayley, 1955) and of H. halobium (Brown, 1965) consisted of lipoprotein.

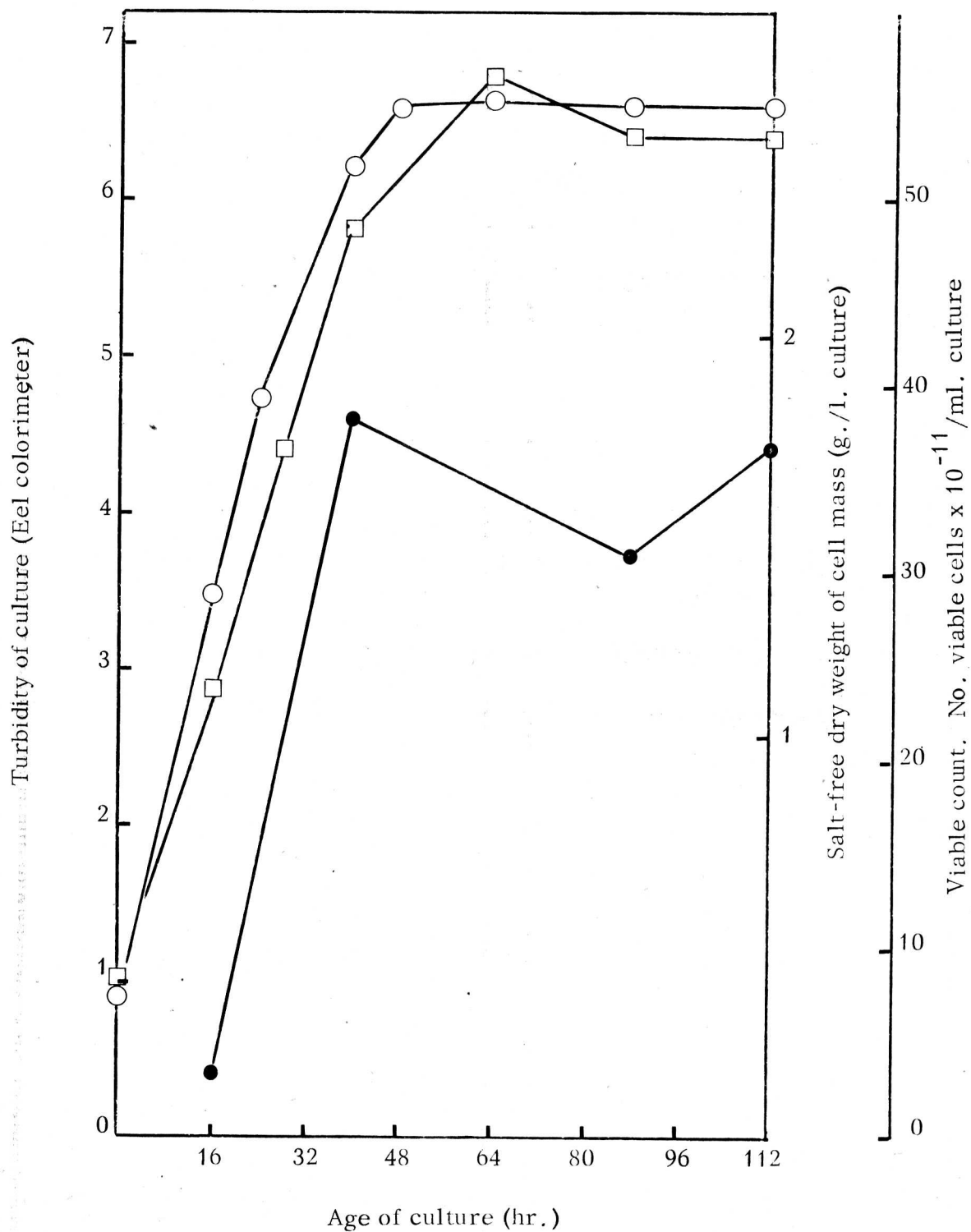
The basic approach used for the analytical fractionation of the complex lipid mixture was a combination of DEAE-cellulose column chromatography with thin-layer chromatography (Rouser, Galli, Lieber, Blank & Privett, 1964) although some subsidiary column work was done for the fractionation of non-polar lipids (see sections 2.17. and 2.18.).

FIG. 3.1.

Growth characteristics of *H. salinarium*

Key :

- turbidity of culture measured in an Eel colorimeter, filter 608, 5 ml. tubes ;
- salt-free dry weight of the cell mass, g. /l. culture ;
- viable count, no. of viable cells $\times 10^{-11}$ /ml. culture.



3.2. Growth characteristics of the cell culture

A growth curve for the cell culture is given in Fig. 3.1. A plot of turbidity or of weight yield of harvested cells against age of culture (which are superimposable) both indicate that there is no observable lag phase. The turbidity of the culture was measured in an Eel colorimeter (filter 608, 5 ml. cells) at 30° using a blank of buffer α . Corrections were made for non-linearity of the scale above about 5 units. The end of the logarithmic phase of growth is at about 48 hr. and the beginning of the stationary phase at about 64 hr.

Throughout the whole of the growth curve (up to 160 hr. growth) the cells remain as slender rods. The appearance of club-shaped or spherical forms was assumed to be indicative of an unhealthy culture, and such cultures were discarded. No change in cell size could be detected at any stage, although estimation of cell volumes by conventional light microscopy is subject to considerable error (Painter & Marr, 1968). If anything, the cells were slightly smaller in older cultures, and there was possibly a higher proportion of very long cells in very young cultures (between 0 and 16 hr.).

The viable count is also given in Fig. 3.1. The viable count was estimated by the method of Miles and Misra (Cruickshank, 1965). Serial dilutions of the cultures in buffer α were drop-counted on agar plates having the same composition as the culture medium (see section 2.2.) with the addition of 1% (w/v) Oxoid agar CM3.

The endogenous activity of washed cells (see section 2.25.) reaches a maximum at the end of the exponential phase and then declines rapidly (Fig. 3.2.).

3.3. Chemical composition of whole cells and the membrane-rich fraction (NM)

Salt-free dry weights, protein and total lipid were estimated in samples of whole cells or in preparations of NM (section 2.5.) as described in 'Methods' (sections 2.6., 2.7. and 2.14) and the results obtained are presented in Figs. 3.3. and 3.4. Fig. 3.3. gives, in addition, the yield of NM as a percentage of the whole cell dry weight. These values have been corrected by the use of carotenoid as a membrane marker; the carotenoid is completely recoverable

FIG. 3.2.

**Endogenous activity of *H. salinarium* as a
function of the age of the culture**

**The cells were washed free from medium, resuspended
in buffer α and the oxygen uptake measured using an
oxygen electrode (section 2.25.)**

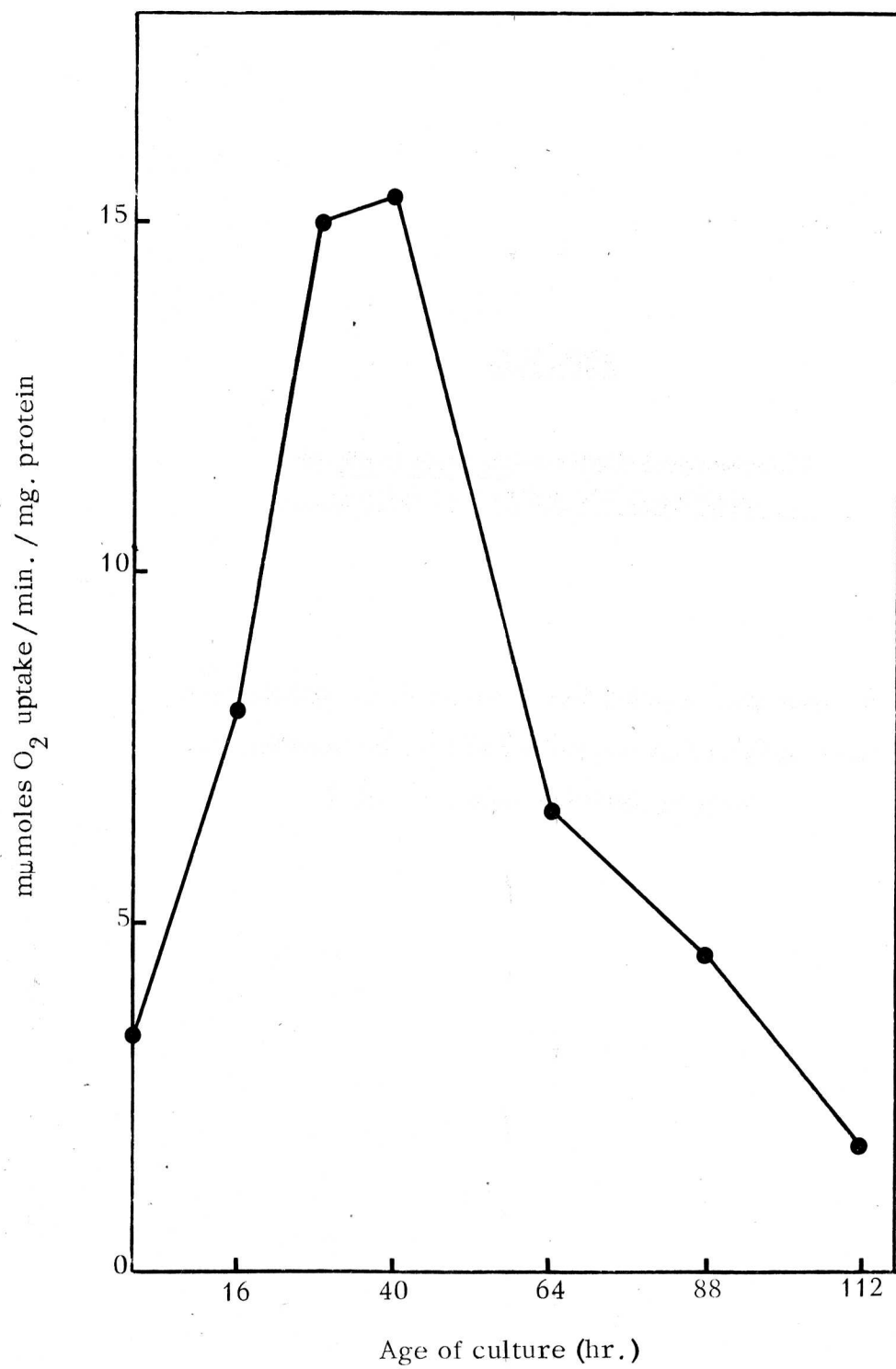


FIG. 3.3.

Composition of whole cells of *H. salinarium*
as a function of age of culture.

Values are expressed as a percentage of the
salt-free dry weight of the whole cells.

Key :

- protein ;
- ▲ lipid ;
- membrane fraction dry weight.

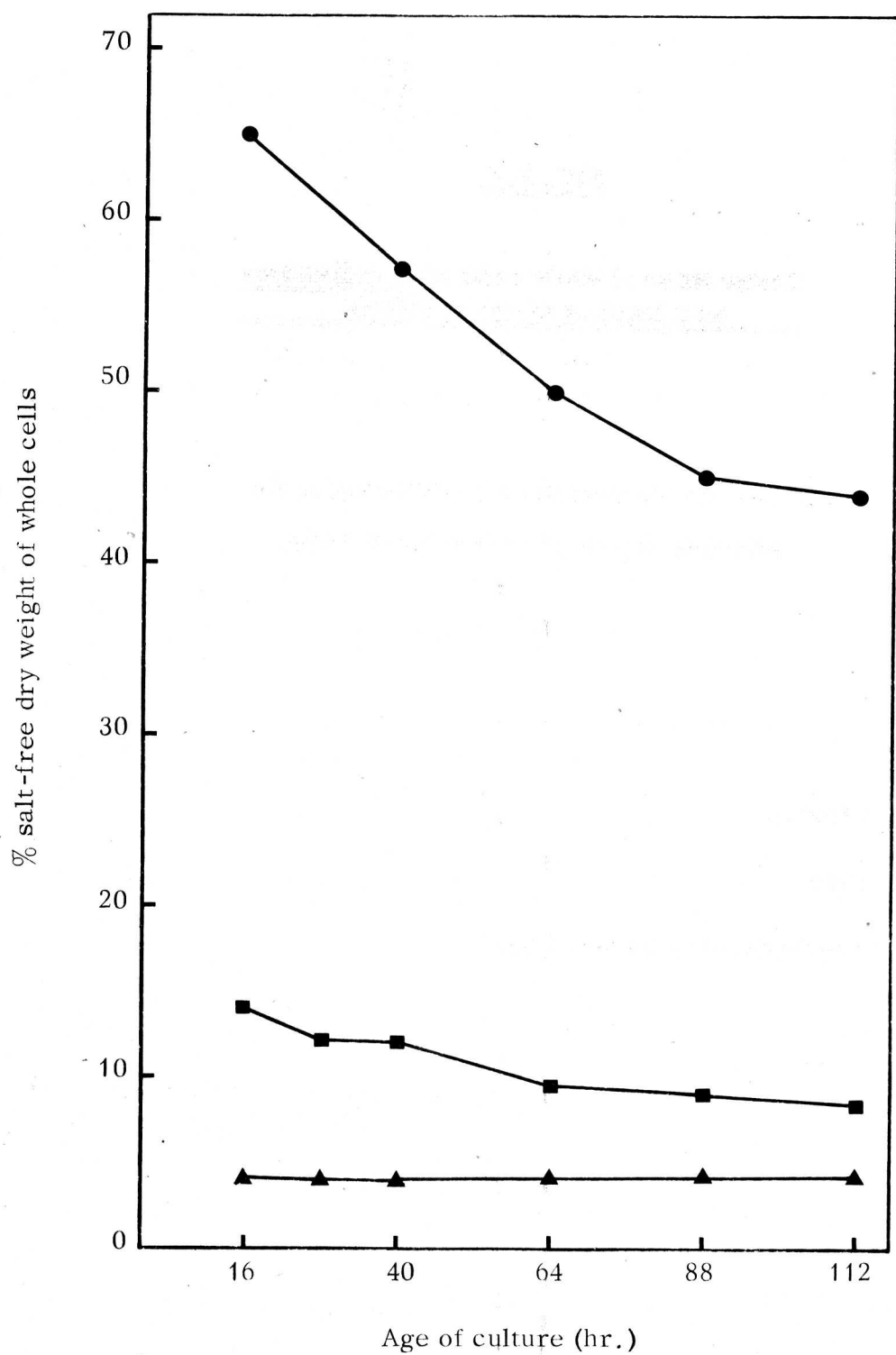


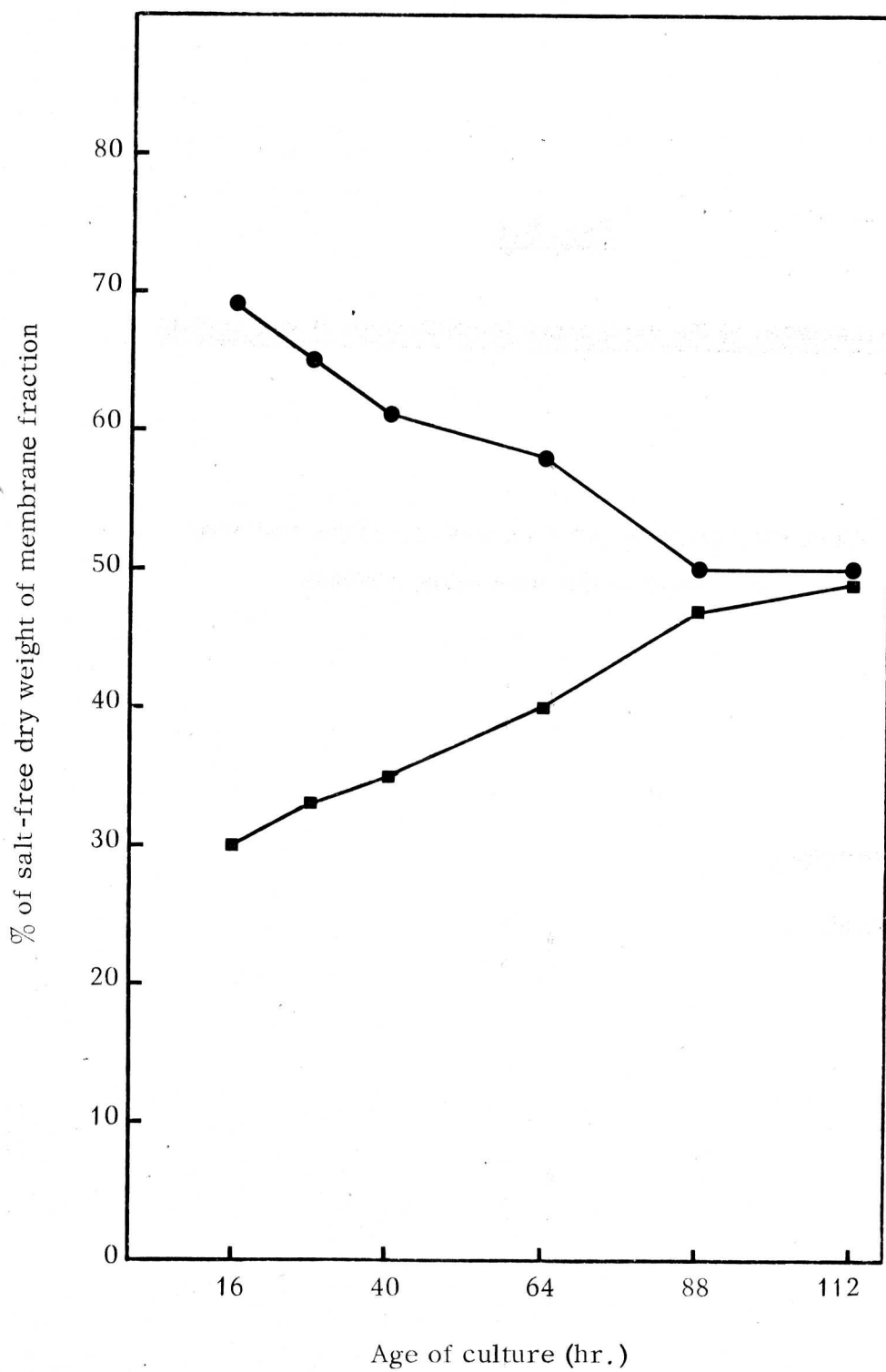
FIG. 3.4.

Composition of the membrane fraction from H.salinarium

**Values are expressed as a percentage of the salt-free
dry weight of the membrane fraction.**

Key :

- protein ;
- lipid.

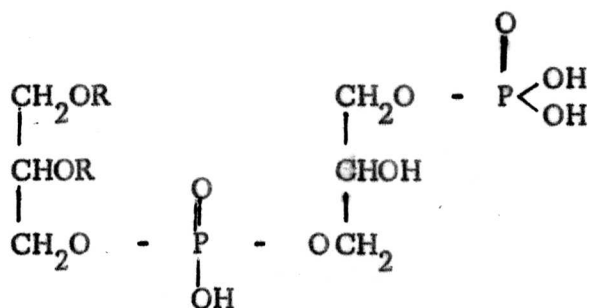


in the membrane fraction after prolonged centrifuging, but for routine analysis a correction for unsedimented membrane material was made on the basis of the carotenoid content in the supernatant.

It is seen in Table 3.1. that if both the total cell lipid and the lipid extracted from membranes are compared to the salt-free dry weight of the cells, then the percentages of lipid present are the same in each case. This indicates that all the major lipids of the cell are concentrated in the membrane. Because of this, lipid analyses were generally performed on lipids extracted directly from whole cells.

3.4. The lipids of *H. salinarium*

Non-saponifiable lipid (section 2.14.) amounted to 78% by weight of the total lipid, the major fraction of which (eluted with 8 - 12% v/v diethyl ether in light petroleum from an alumina column, section 2.18.) had an i.r. spectrum (Fig. 3.5.) identical with that reported for 2,3-di-O-dihydrophytylglycerol. This component has previously been observed in non-saponifiable fractions from several species of *Halobacterium*, including a strain of *H. salinarium* (Kates, et.al., 1966). It was also reported (Kates, Yengoyan & Sastry, 1965) that the di-O-dihydrophytylglycerol was derived mainly by hydrolysis from a di-ether analogue of glycerophosphoryl glycerophosphate (I) (R = 3,7,11,15-tetramethylhexaheptyl, or dihydrophytyl).



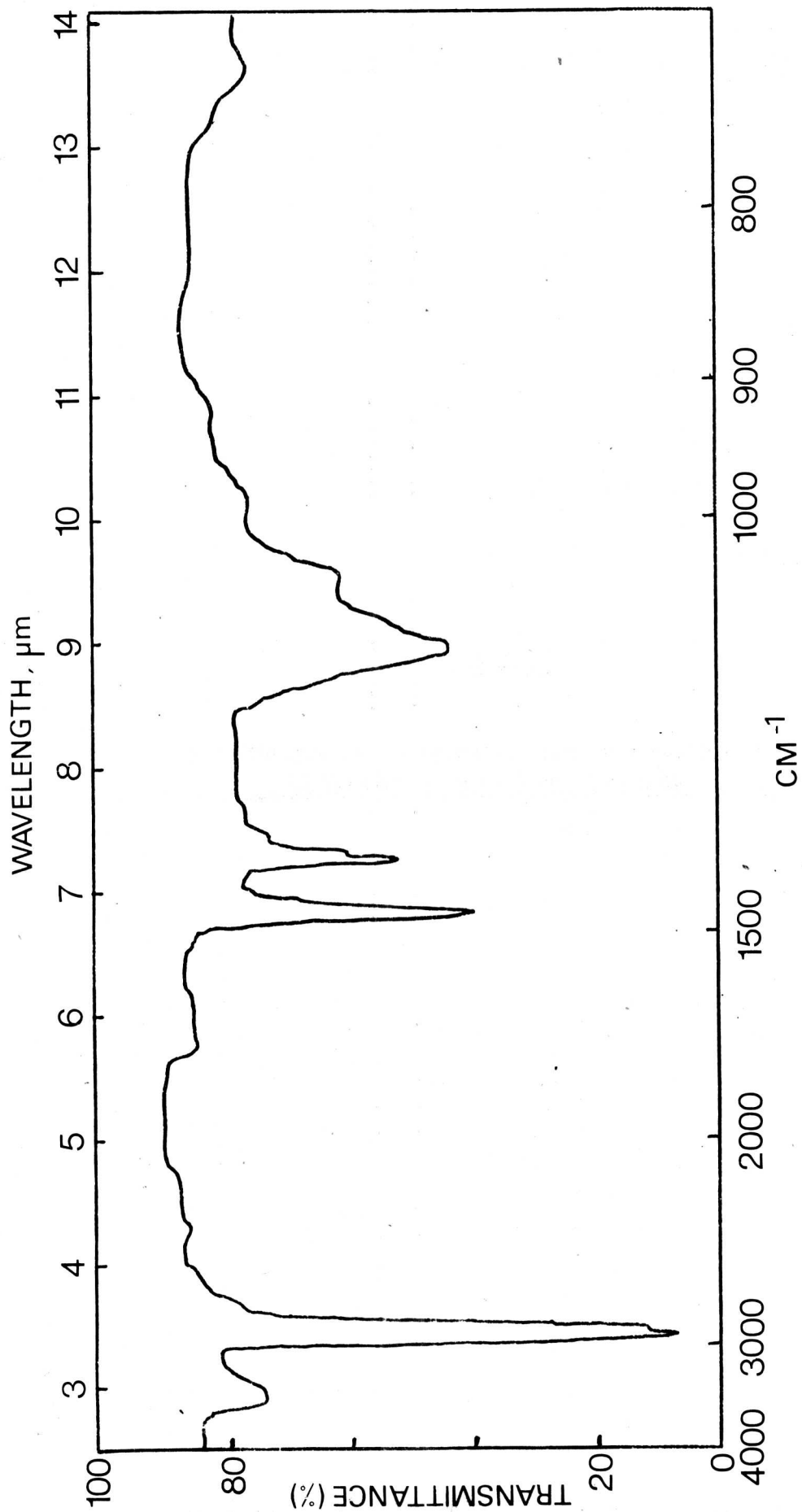
I

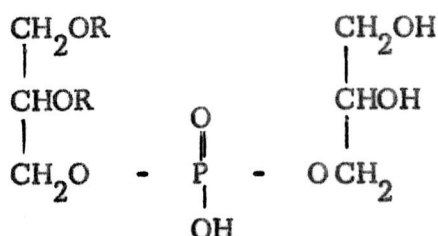
The di-ether analogue of
glycerophosphoryl glycerophosphate

This compound in *H. cutirubrum* accounts for 73% by weight of the total lipid (Sehgal, Kates & Gibbons, 1962). Fauré, Maréchal & Troestler (1964) reported the presence of a minor phospholipid, a di-ether analogue of glycerophosphoryl glycerol (II). (R = dihydrophytyl).

FIG. 3.5.

**I.r. spectrum of major fraction of non-saponifiable
lipid extracted from *H. salinarium***





II

The di-ether analogue of
glycerophosphoryl glycerol

The preliminary evidence therefore suggests that the lipids of H. salinarium, strain 1, might well be similar to those of other Halobacteria, in particular in having ether-linked polyisoprenoid side chains rather than the more usual ester-linked fatty acid side chains.

The chromatography on DEAE-cellulose of the total cell lipid from H. salinarium indicated that the majority of the lipid, amounting to approximately 93% of the total, was polar, in all probability, phospholipid. It was eluted with the chloroform-methanol-ammonia mixture mentioned in 'Methods' (section 2.16.). The remainder of the lipid, the non-polar fraction, was eluted with chloroform and contained none of the lipid phosphorus. Occasionally, some lipid (up to 5 - 6% of the total lipid) was eluted with the chloroform-methanol mixture (2:1, by vol.), but this component was also present in the major polar fraction (eluted with the chloroform-methanol-ammonia mixture), as judged by thin-layer chromatography of the fractions in solvent system A (see section 2.19.). No lipid was detected in any other fractions eluted from the DEAE-cellulose column if a more complicated elution scheme, which included other solvent combinations, was employed.

On thin-layer chromatography, the non-polar fraction of the total lipid migrated near the solvent front in solvent system A, as would be expected (spot 7 in Fig. 3.6.). In general, therefore, for the analysis of the polar lipids of H. salinarium, the total lipids were run on thin-layer chromatoplates, and the spot at the solvent front ignored. A chromatogram of total lipids is presented diagrammatically in Fig. 3.6., together with an analysis of an extract from H. cutirubrum for comparison.

A tentative identification only can be made for the various fractions of

TABLE 3.1.Distribution of lipid in whole cells and membranes

<u>Age of culture (hr.)</u>	<u>% Total cell salt-free dry weight</u>		<u>Membrane lipid % of total lipid</u>
	cell lipid	membrane lipid	
16	4.1	4.2	102
28	4.0	3.9	98
40	4.1	4.2	102
64	4.2	3.8	90
88	4.3	4.3	100
112	4.2	4.3	102
			av. 99%

FIG. 3.6.

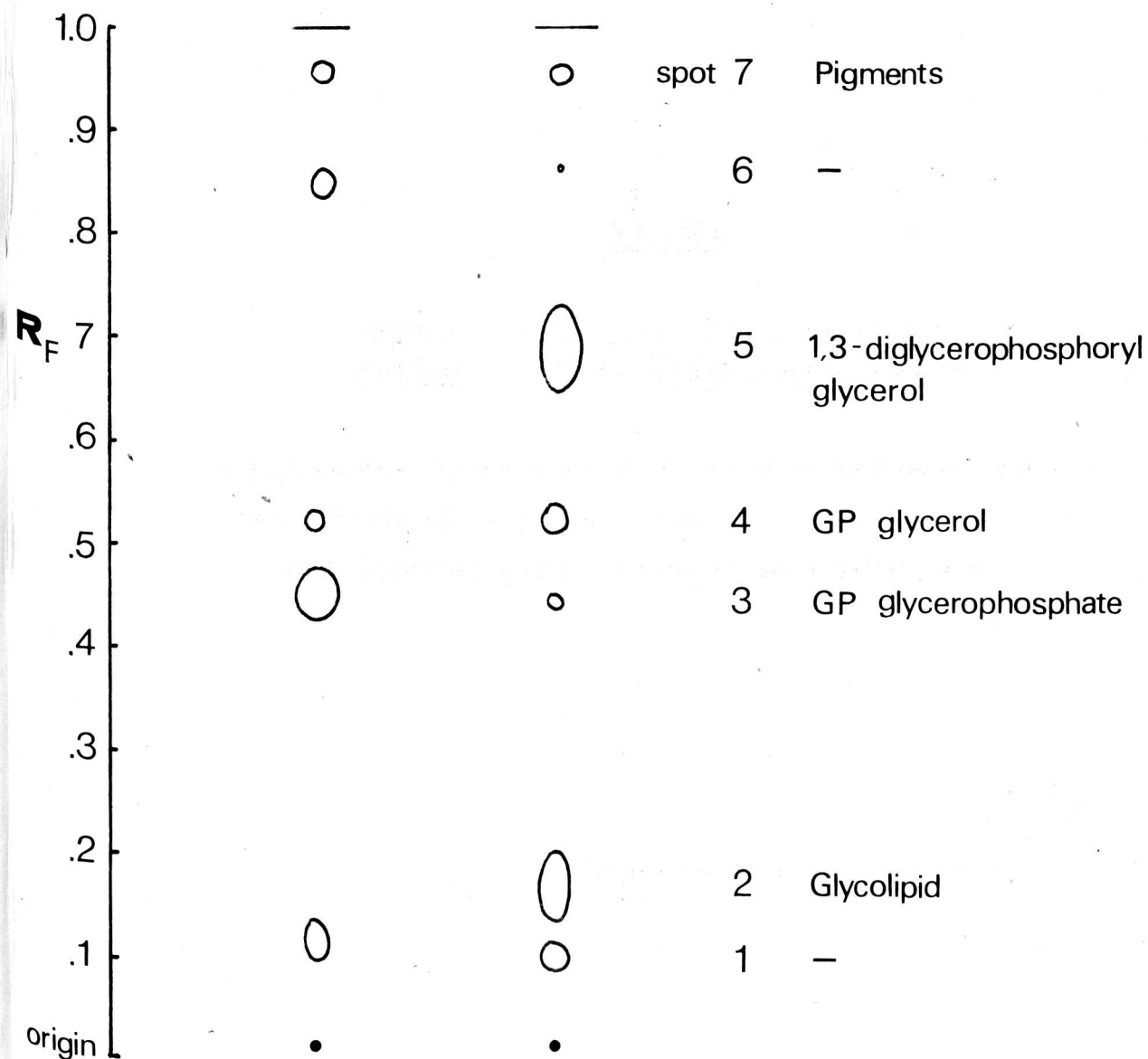
**Thin-layer chromatography of the total lipids
extracted from H. salinarium and H. cutirubrum**

Chromatoplates were developed in solvent system A (section 2.19.).

**Tentative identification of the spots are given. The phospholipids
are ether analogues of the normal glycerophosphoryl lipids.**

Key :

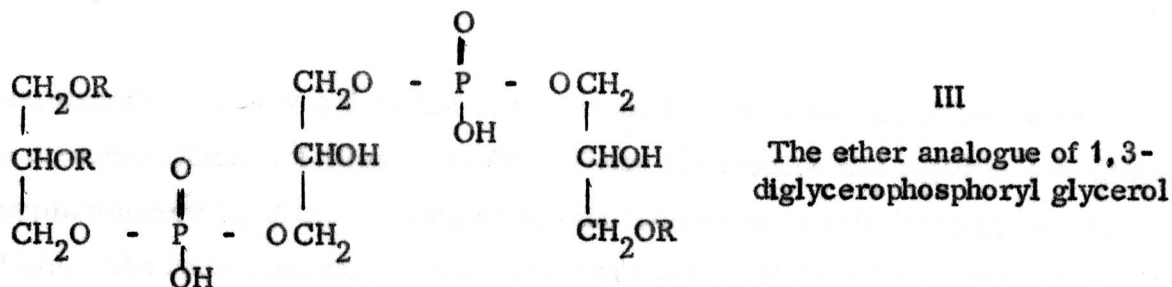
GP glycerophosphoryl (ether analogue)

H. cutirubrumH. salinarium

polar lipid separated by thin-layer chromatography, as no dihydrophytyl ether lipids were available as standards. Of the standards which were available (from Sigma), glycerophosphoryl choline, glycerophosphoryl ethanolamine, glycerophosphoryl glycerol and glycerophosphoryl inositol, only a spot corresponding to glycerophosphoryl glycerol could be detected in chromatograms of H. salinarium lipids (Fig. 3.6., spot 4). This was the spot which appeared in thin-layer chromatograms of the chloroform-methanol eluate (as the only observable component, see above). It was periodate-Schiff positive, and was tentatively identified as the ether analogue of glycerophosphoryl glycerol (II). The content of this component in the total lipid, as judged by the DEAE-cellulose chromatography (5 - 6% by weight in the chloroform-methanol eluate, slightly less in the chloroform-methanol-ammonia eluate) is consistent with the value obtained directly by periodate oxidation of the total lipid, followed by an estimation of liberated formaldehyde (section 2.15.), which was 12%, by weight, of the total lipid.

Of the other spots on the thin-layer chromatograms of total lipids (Fig. 3.6.), tentative identifications are as follows : Spot 2 shows reducing properties with a 1% silver nitrate spray and its R_F of 0.18 corresponds with that of glycolipid sulphate (Kates, et.al., 1967). Spot 3, being the only spot giving a blue colour with rhodamine 6G, and corresponding with the major spot of H. cutirubrum lipids, is tentatively identified as glycerophosphoryl glycerophosphate (ether analogue, I).

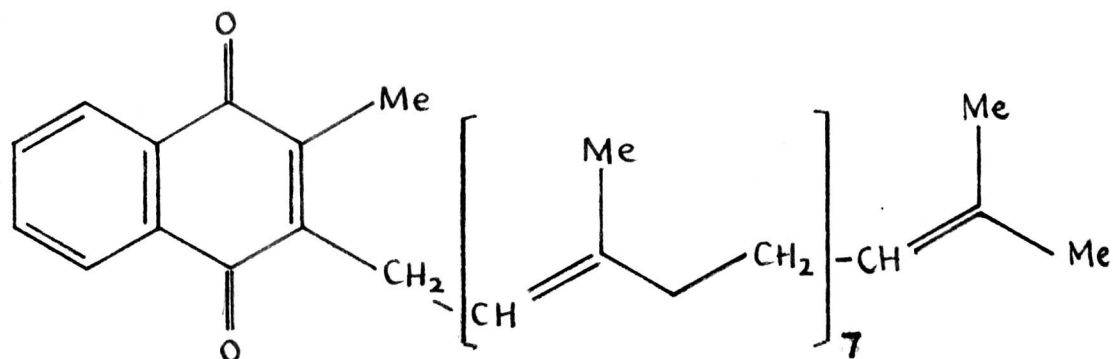
Spot 5, which is absent in H. cutirubrum, constitutes a major lipid component of H. salinarium. It is periodate-Schiff negative, gives a positive reaction with the Hanes and Isherwood phosphate spray, and gives an orange colour with rhodamine. The spot is tentatively identified as the ether analogue of 1,3-diglycerophosphoryl glycerol (III). (R = dihydrophytyl).



The presence of a component with the structure of pyro-glycerophosphoryl glycerophosphate, i.e. a dimer of I with a pyrophosphate linkage, as suggested by Fauré, Maréchal & Troestler (1963) was considered unlikely since there was no pyrophosphate absorption band in the i.r. spectrum of the total lipid (Fig. 3.7.).

It was not found possible to isolate from extracts of *H. salinarium* glycerophosphoryl glycerophosphate (I) as the barium salt as described by Kates, *et.al.*, (1965). A barium salt was obtained from *H. cutirubrum* extracts, which may be that of glycerophosphoryl glycerophosphate (I). (Found : P = 4.6% ; $C_{46}H_{93}O_{11}P_2Ba_{3/2}$ requires P = 5.69%). An i.r. spectrum is presented in Fig. 3.8.

Of the non-polar lipids, which were eluted from the DEAE-cellulose column by chloroform, two components had been previously characterised. The menaquinone which could be extracted from the organism was shown to have an identical R_F value to that of an authentic sample of menaquinone-8 (IV, Vitamin K_2 -40) on reversed phase paper chromatography by Stevenson (1965). The structure of the



IV. Menaquinone - 8

carotenoid present was determined by Liaaen Jensen (1962) and shown to be a di-demethylated spirilloxanthin (V). These two components together constituted approximately 3% of the total lipid, and therefore nearly half of the non-polar lipid. The remainder (approximately 4% of the total lipid) of the non-polar lipids

FIG. 3.7.

**I.r. spectrum of total lipids
extracted from *H. salinarium***

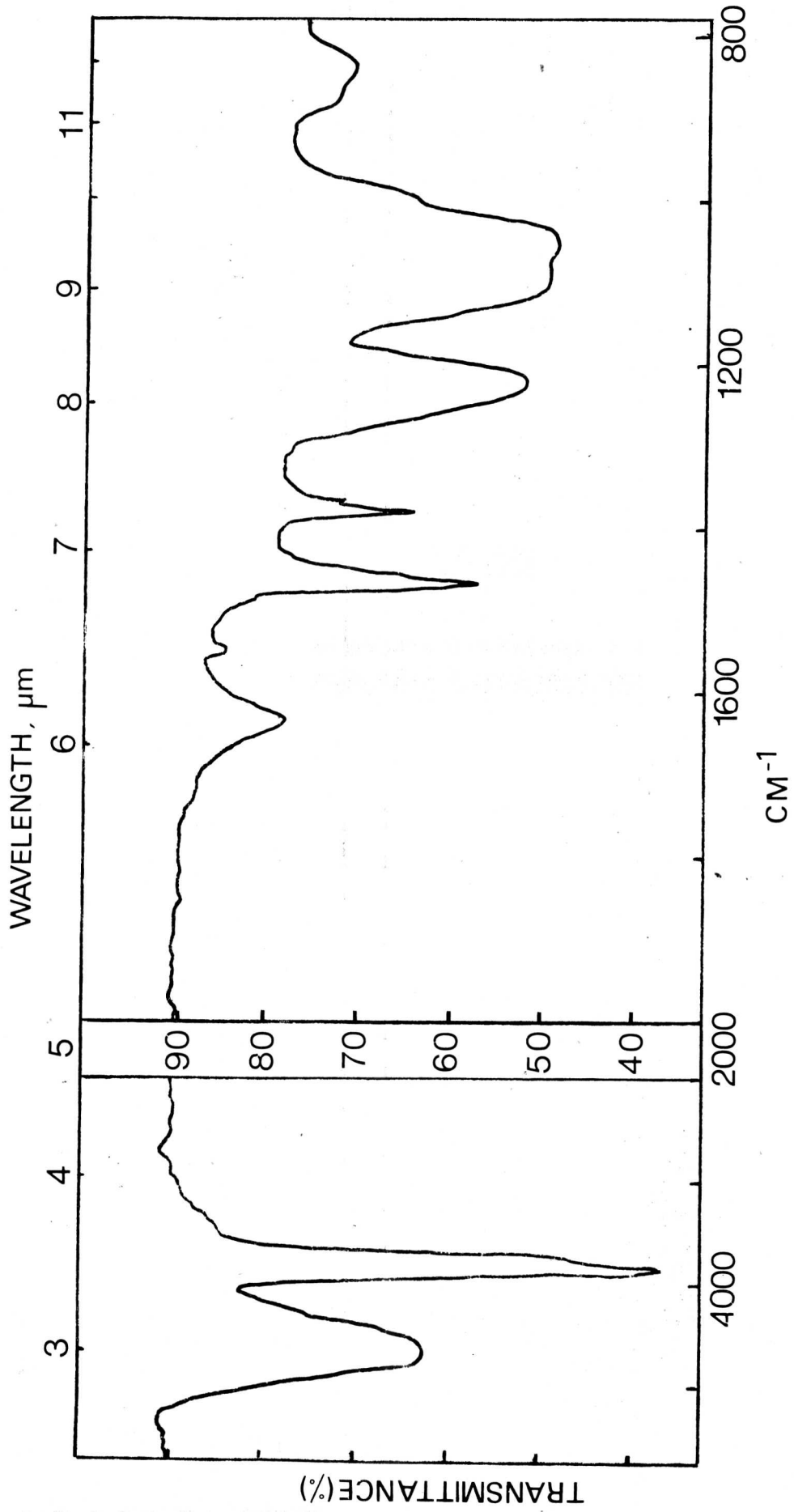
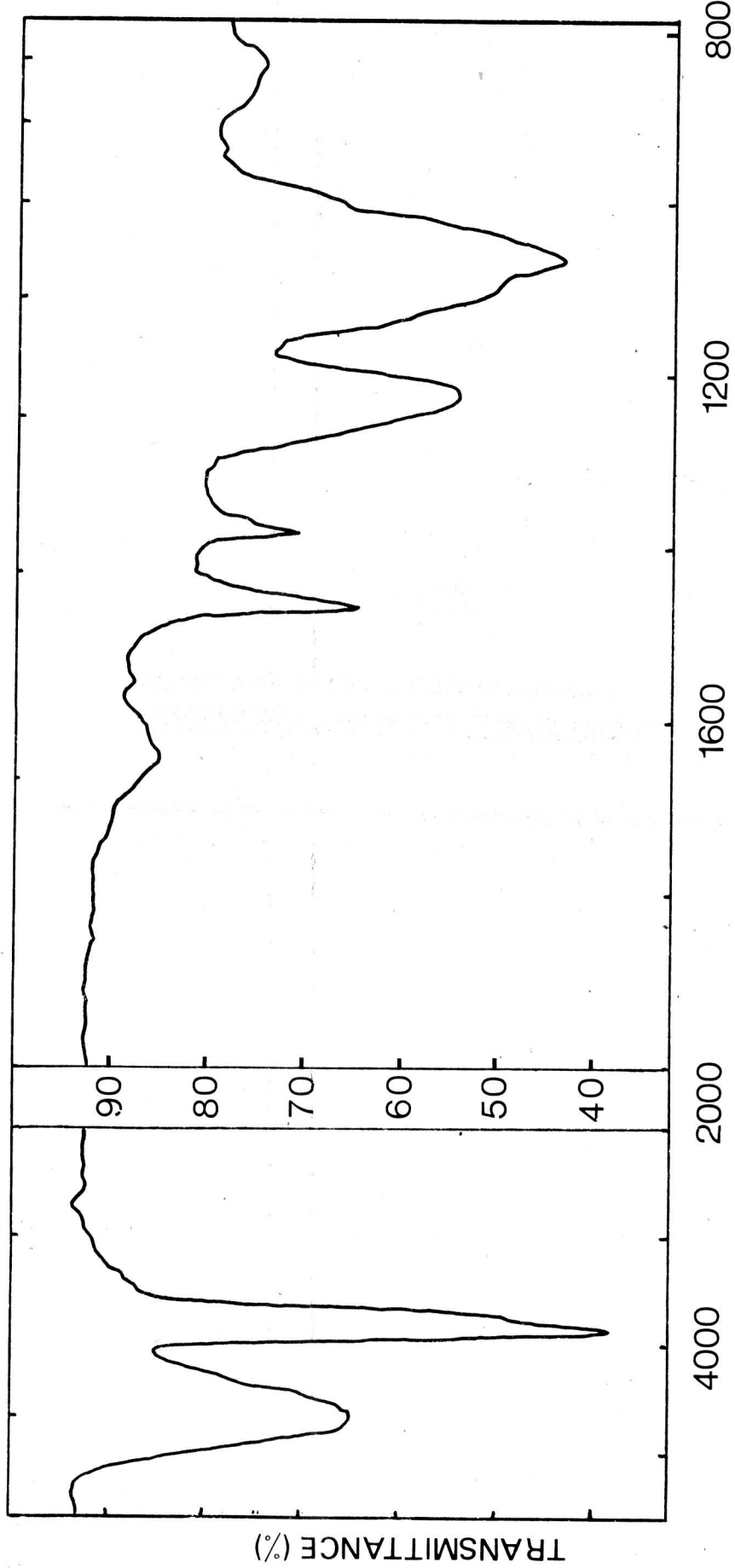


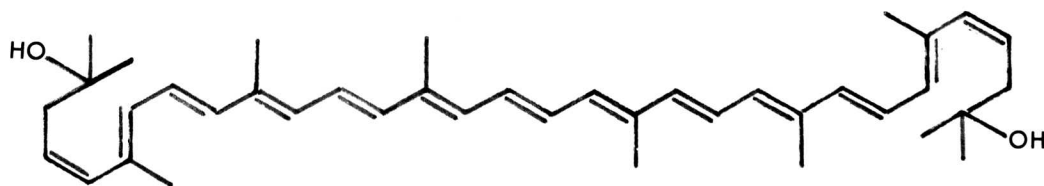
FIG. 3.8.

**I.r. spectrum of barium salt of phospholipid
obtained from *H.cutirubrum* lipid extracts**

The barium salt was obtained as described in section 3.4.

WAVELENGTH, μm





V. α - bacterioruberin

from the DEAE-cellulose column were eluted by 40 - 60° light petroleum from the Decalso column used for the purification of menaquinone (see section 2.19.). The i.r. spectrum of this material indicated that it was primarily hydrocarbon (Fig. 3.9.). On thin-layer chromatography (see section 2.17.) using solvent system B and visualising lipid spots with a sulphuric acid spray, the major component had an R_F value of 0.85 (Fig. 3.10.). The light petroleum eluate from the Decalso column was coloured pale yellow, this colour probably being due to an unidentified pigment, possibly a carotenoid precursor. A u.v. spectrum is recorded in Fig. 3.11. U.v. spectra for the menaquinone- and carotenoid-containing fractions (see section 2.19.) are presented in Figs. 3.12 and 3.13. The latter fraction, eluted from the Decalso column with methylal, was further chromatographed on thin-layer plates (see section 2.17.) in solvent system C. A chromatogram is presented diagrammatically in Fig. 3.14. Two yellow spots moved ahead of the carotenoid band. These were probably degradation products of the carotenoid, as their concentration markedly increased if the lipid samples were heated for only a few minutes when dry.

3.5. Variations in the lipid composition with the growth phase

Thin-layer chromatograms of total lipids extracted from whole cells and from membrane-rich fractions were indistinguishable and showed no variation with the age of the culture. Although no changes in the quantities of the various major lipid components of young and old cells could be detected visually, it was hoped that it would be possible to make a more quantitative comparison. Attempts were

FIG. 3.9.

**I. r. spectrum of non-polar lipids obtained
from H. salinarium lipid extracts**

**The non-polar lipids were obtained in the light petroleum eluate
from the Decalso column fractionation (section 2.17.).**

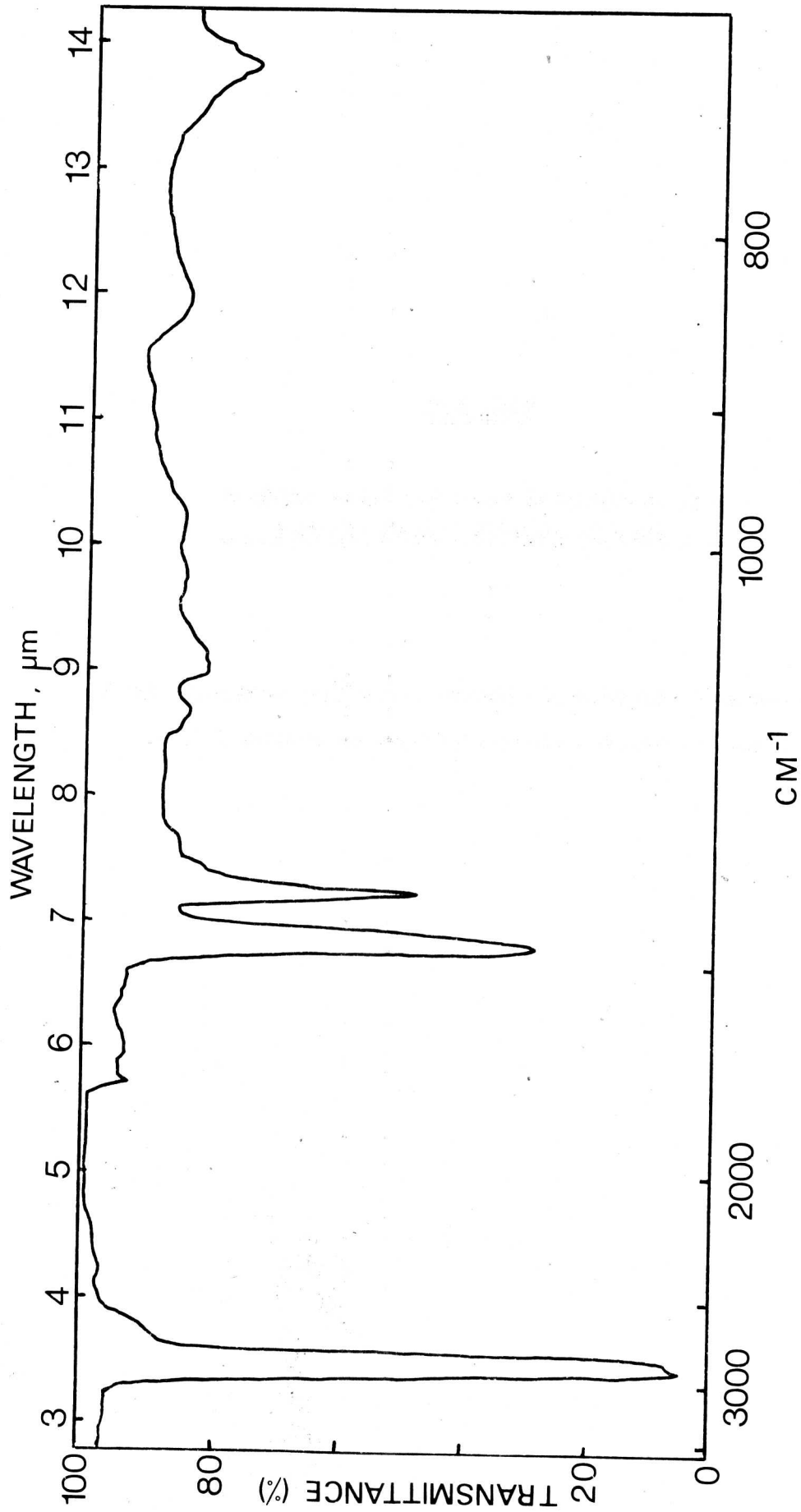


FIG. 3.10.

**Thin-layer chromatography of non-polar lipids
obtained from *H. salinarium* lipid extracts**

**The non-polar lipids were obtained in the light petroleum eluate
from the Decalso column fractionation (section 2.17.). The
chromatoplate was developed in solvent system B
(section 2.19.).**

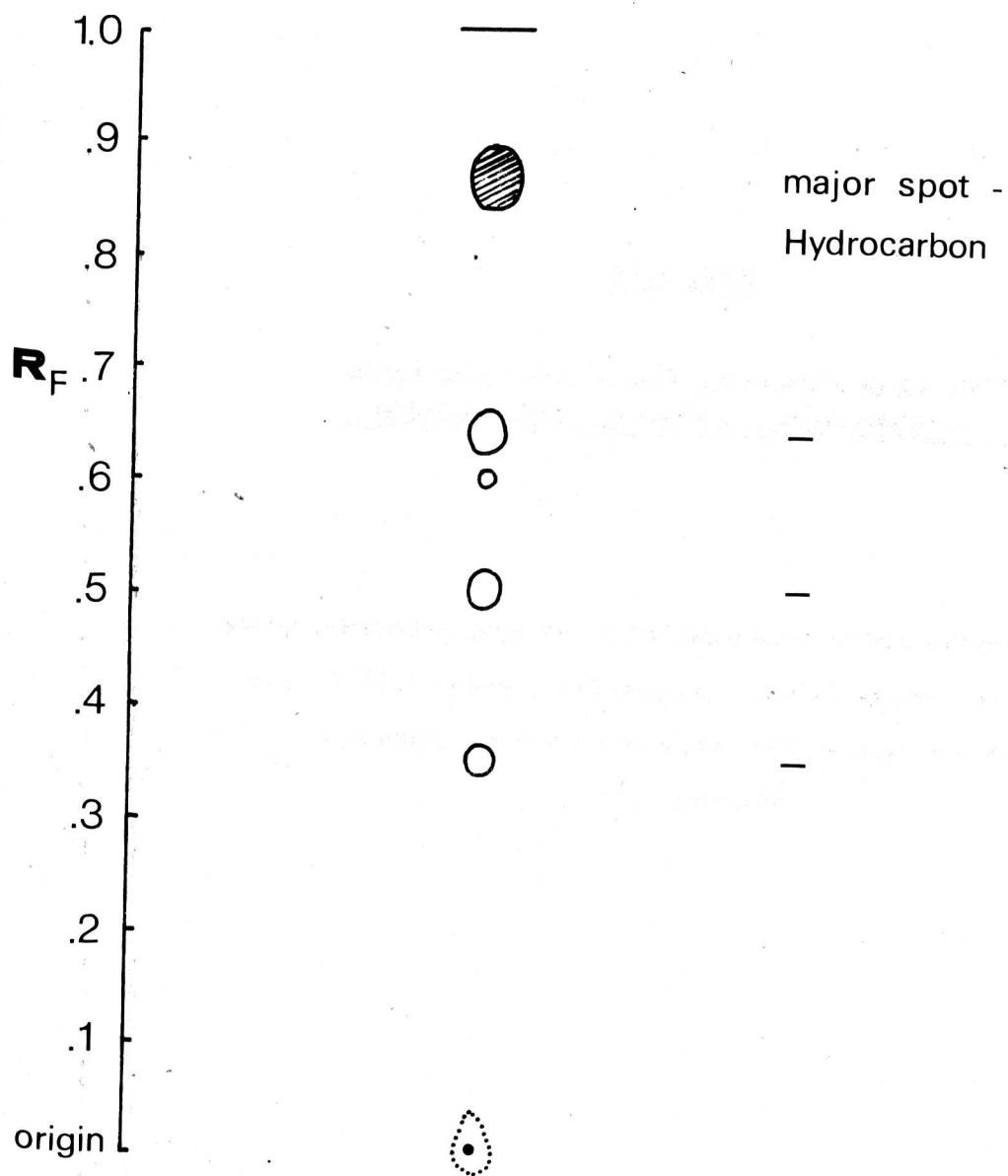


FIG. 3.11.

**U.v. spectrum of non-polar lipids obtained
from *H. salinarium* lipid extracts**

**The non-polar lipids were obtained in the light petroleum eluate
from the Decalso column fractionation (section 2.17.). The
spectrum was taken in cyclohexane solution.**

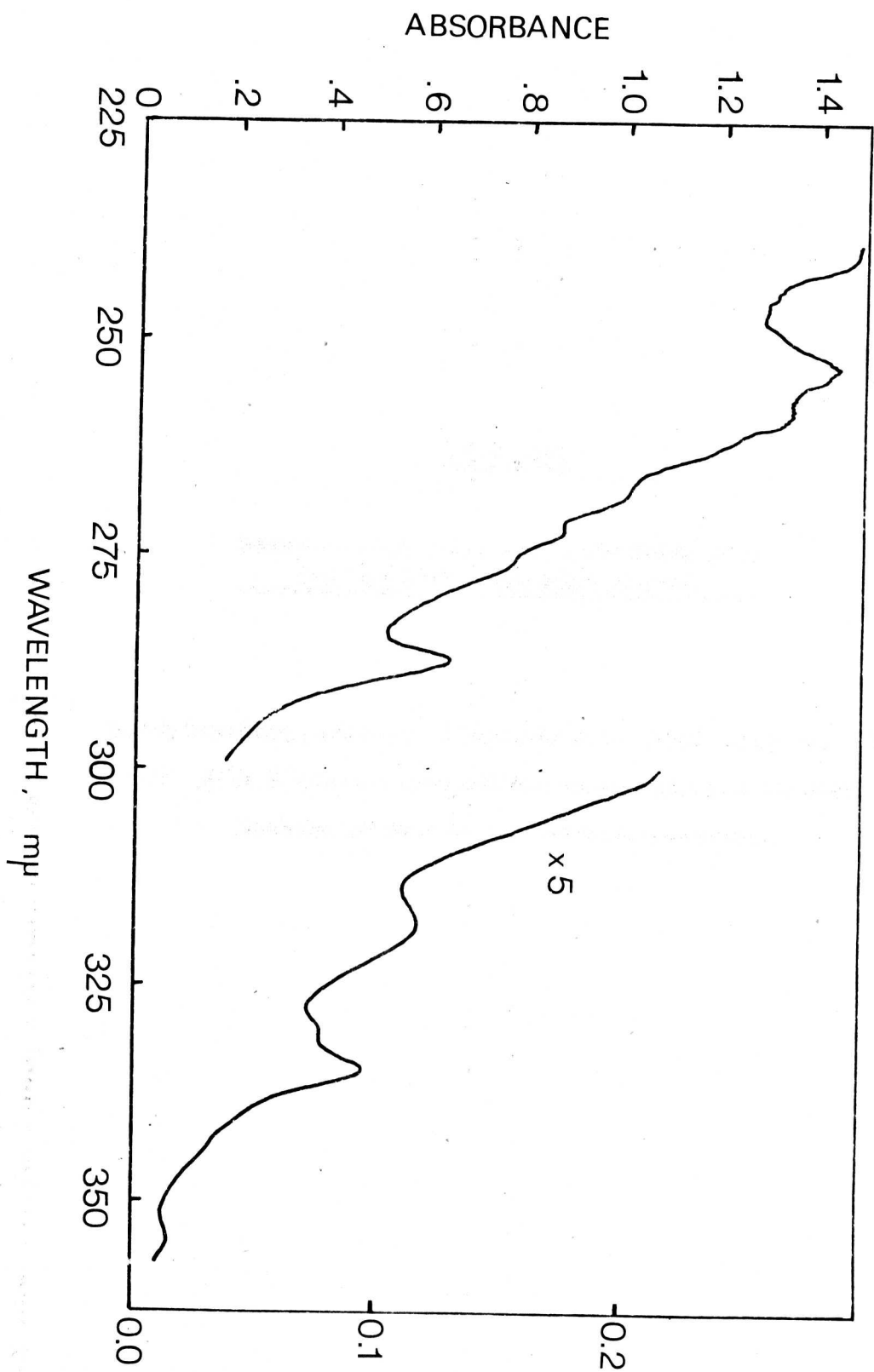


FIG. 3.12.

**U.v. spectrum of menaquinone-containing fraction
obtained from *H. salinarium* lipid extracts**

The fraction was obtained in the 4% (v/v) diethylether in
light petroleum eluate in the Decalso fractionation of
non-polar lipids (section 2.17.).

Key :

- spectrum taken in cyclohexane solution ;
- spectrum taken in alcoholic solution ;
- spectrum of sample after reduction with sodium borohydride (in alcoholic solution).

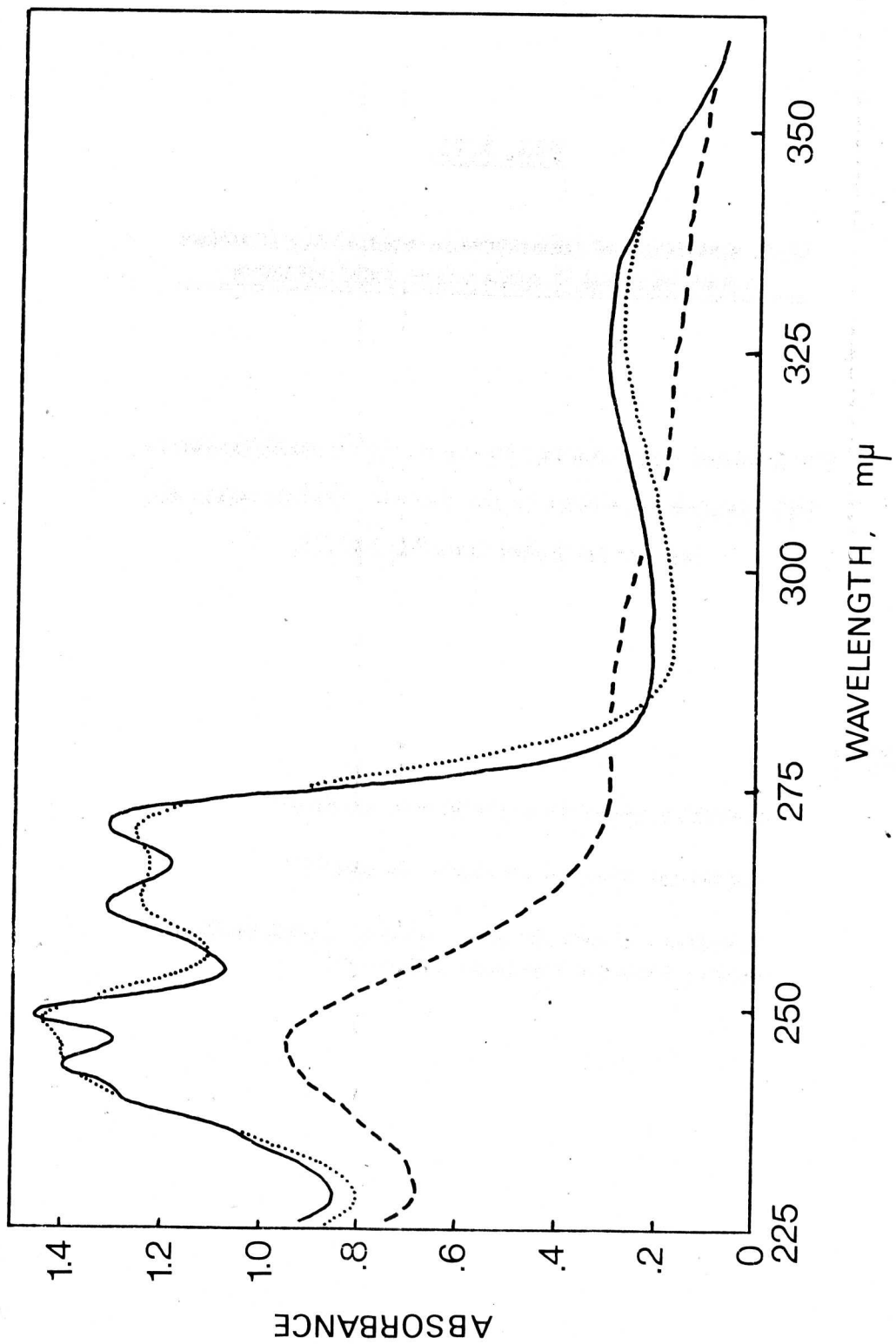


FIG. 3.13.

**U.v. spectrum of carotenoid-containing fraction
obtained from H. salinarium lipid extracts**

**The fraction was obtained in the methylal eluate in the Decalso
fractionation of non-polar lipids (section 2.17.). The
spectrum was taken in chloroform solution.**

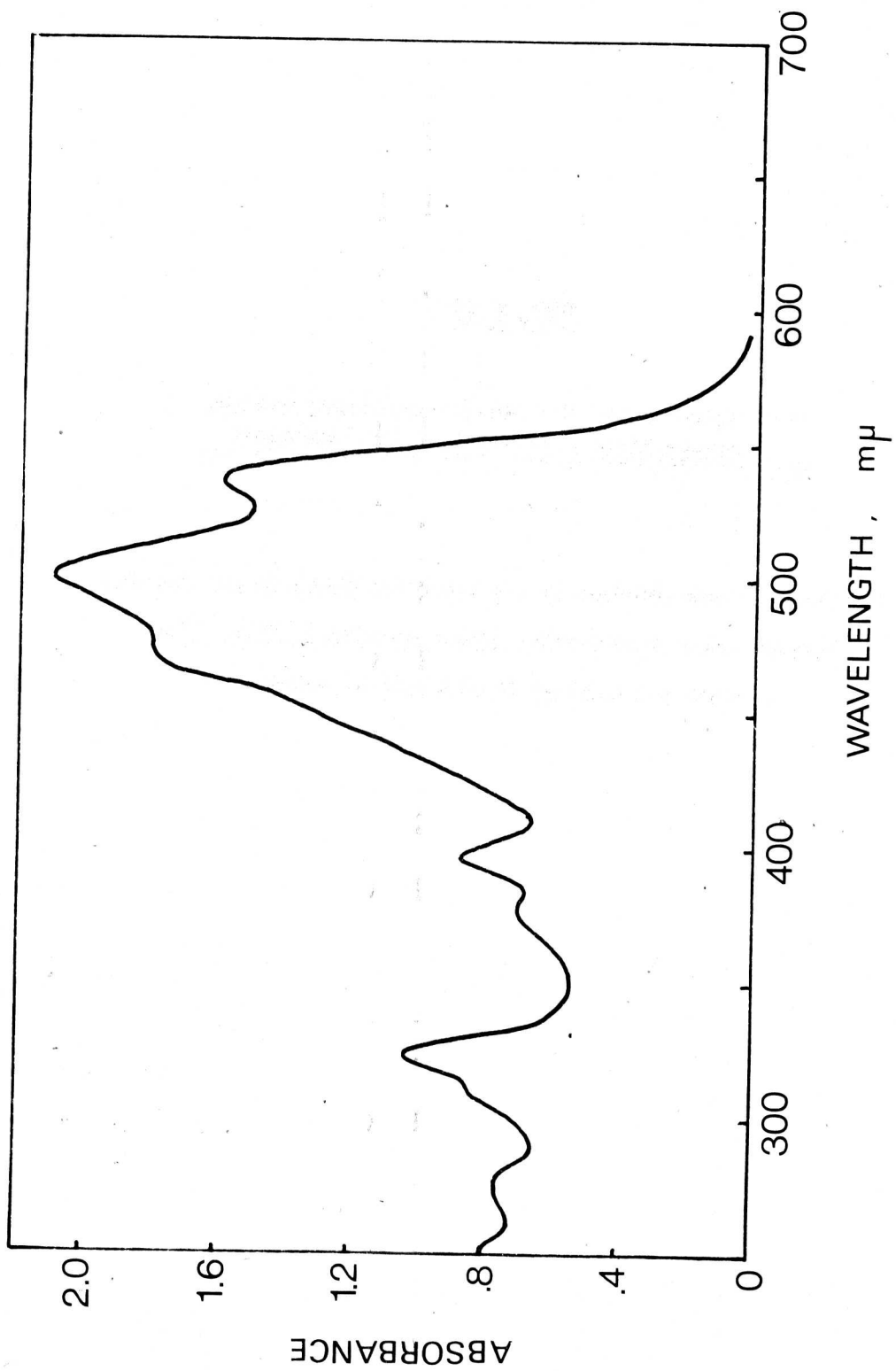
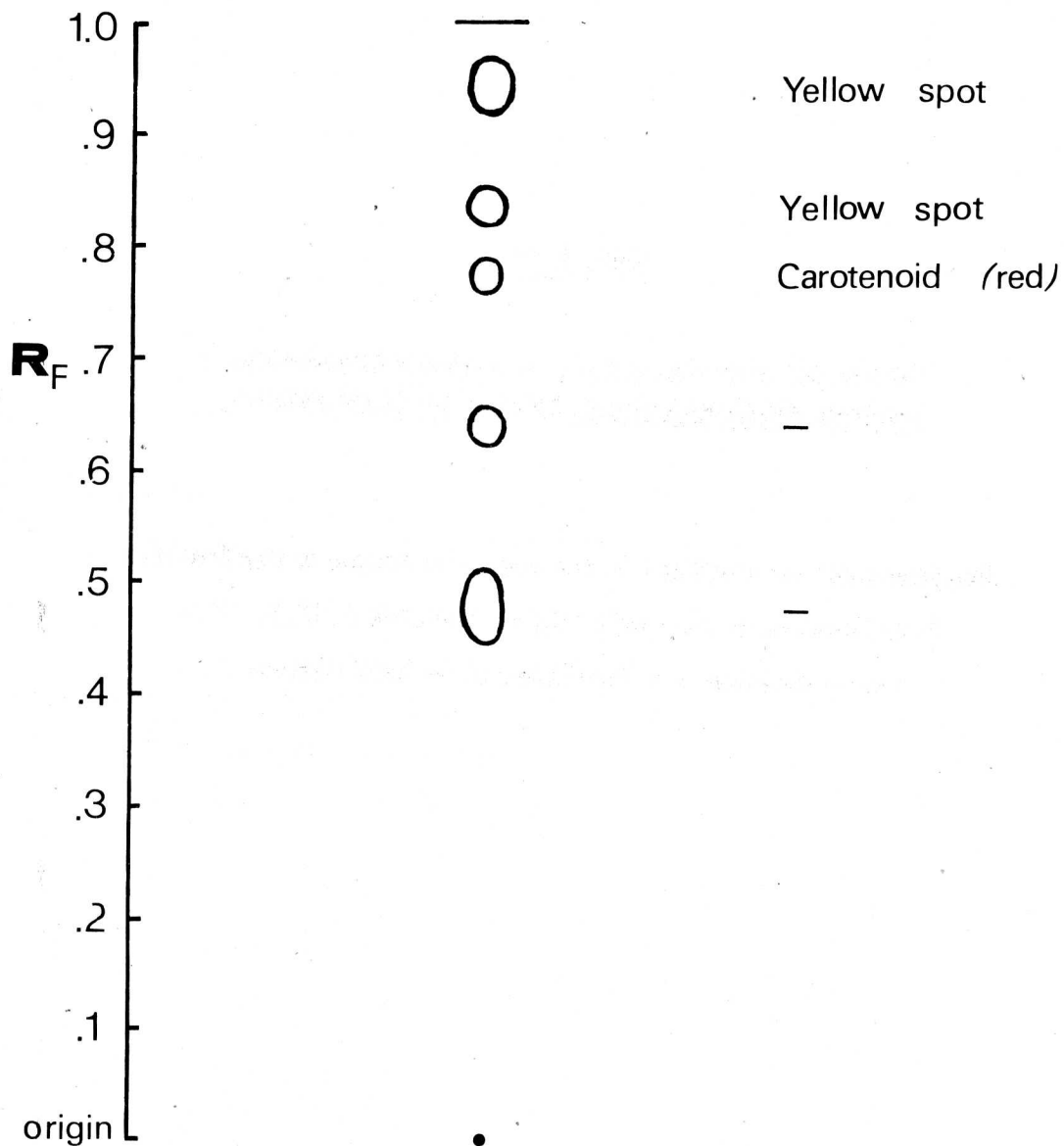


FIG. 3.14.

**Thin-layer chromatography of carotenoid-containing
fraction obtained from H.salinarium lipid extracts**

**The fraction was obtained in the methylal eluate in the Decalso
fractionation of non-polar lipids (section 2.17.). The
chromatoplate was developed in solvent system C.**



therefore made to estimate the phosphorus content of the fractions obtained by thin-layer chromatography of the total lipids by scraping sections of the absorbent off the plate and estimating phosphate as described in section 2.15. The chromatoplate and absorbent were thoroughly washed in the developing solvent (system A) before activation by placing them in the chromatography tank such that the upper edge of the chromatoplate protruded 1 cm. above the ground glass upper surface of the tank. The lid of the tank was then replaced such that the smallest possible gap was left between it and the chromatoplate. By this means, the plate was continuously washed with solvent, which evaporated on the exposed area of the plate.

Phosphorus analyses were performed without prior removal of the lipid from the silica gel absorbent (Parker & Peterson, 1965; Rhee & Dugan, 1967). However, using either of the two phosphorus estimations (see section 2.15.) reproducible results could not be obtained.

On the other hand, reproducible results were obtained for phosphorus estimations of total lipid extracts, and these are presented in Table 3.2. The two methods of estimating phosphorus gave comparable results. A value for the phosphorus content of total lipid extracted from H.cutirubrum is given for comparison.

3.6. Variations of the minor lipid components with the growth phase

Two minor components, menaquinone and carotenoid, could be estimated quantitatively with relative ease. Both components were estimated spectrophotometrically after isolation in a relatively pure state (see section 2.20.). The results are presented in Fig. 3.15. It should be noted that the menaquinone and carotenoid rise and fall synchronously, retaining an approximately constant molar ratio one to the other (average molar ratio = 1 mole of carotenoid to 2.08 moles menaquinone).

3.7. Conclusion

The expected variations in the composition of whole cells and of cell membranes of Halobacterium salinarium have been observed (see Fig. 3.3.).

FIG. 3.15

**Variation in the menaquinone and carotenoid content
of the lipid extracted from H. salinarium as a function
of the age of the culture**

Key :

- menaquinone, average of three determinations
(standard error av. 6.6%);
- carotenoid, average of three determinations
(standard error av. 4.7%).

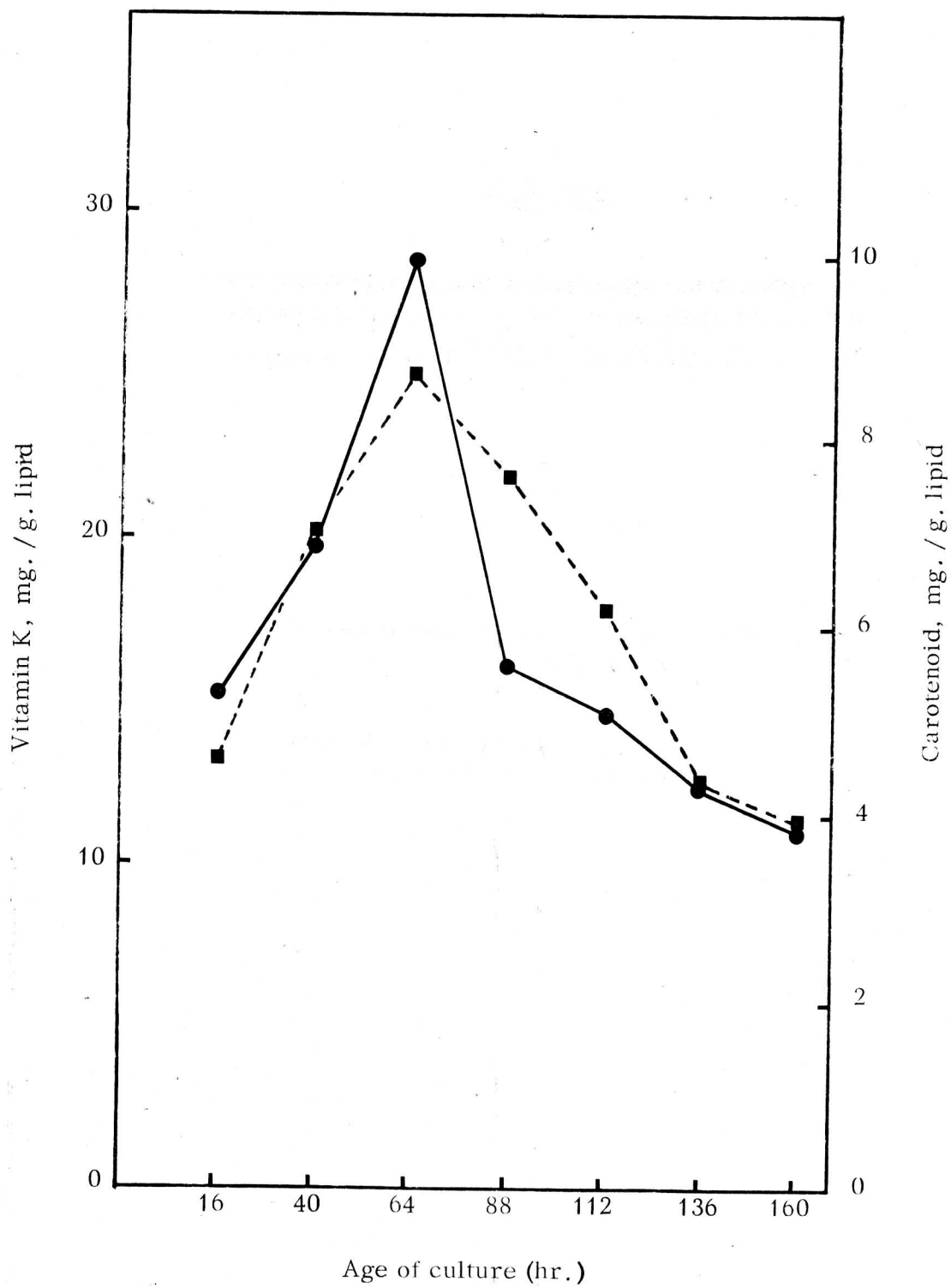


TABLE 3.2.

The analysis of lipid phosphorus

Samples of total lipid extracts were analysed for phosphorus by the methods of Rhee & Dugan or of McClare (see section 2.15.). Each value is the average of 4 determinations (standard error, average, 4.9%).

<u>Organism</u>	<u>Age of culture (hours)</u>	<u>Lipid phosphorus (% total lipid)</u>	
		<u>Rhee & Dugan</u>	<u>McClare</u>
H. salinarium	28	3.75	
H. salinarium	40	3.62	
H. salinarium	64	3.69	3.62
H. salinarium	136	3.80	
H. cutirubrum	64		4.15

A decrease in the proportion of cell membrane to whole cell occurs primarily during the logarithmic phase of growth, and this change, within the limits of experimental error, can be attributed entirely to a decrease in membrane protein. The membrane lipid (as a fraction of whole cell dry weight) remains constant throughout the growth cycle, although when expressed as a fraction of membrane weight, the lipid appears to increase.

The overall decrease in the proportion of membrane during growth could of course be accounted for by an increase in cell volume (due, e.g., to a failure of the cells to divide). However, no change in cell size could be detected visually.

The decrease in membrane must therefore be explained as a progressive decrease in the rate of protein synthesis or as an increase in degradative pathways converting protein into non-proteinaceous compounds. It may be significant that the cell protein as a function of cell dry weight also decreases markedly during the growth phase.

Using the largely qualitative "fingerprinting" technique (see section 3.1.), it was observed that neither the nature of the cell lipids nor their proportions appeared to change appreciably during the growth cycle. The bulk, if not all, of the lipid material is present in the membrane-rich fraction from the lysed cells (fraction NM), and therefore it may be concluded that the lipid composition of the cell membrane does not change appreciably with the phase of growth, the nutritional status or the age of the culture.

The picture that emerges is therefore one of a membrane with a very stable lipid component and a gradually declining protein component.

The behaviour of the menaquinone and carotenoid is more difficult to discuss. As will be seen later (Chapter IV), both components are localised entirely in the membrane together with the cytochromes and other components of the electron transport chain. Menaquinone and the carotenoids are biosynthetically related, but a functional relationship has never been established. The rise in menaquinone and carotenoid content during the first 64 hr. of growth (Fig. 3.15.) and the subsequent fall do not appear to be related in any way to the overall protein-lipid ratio in the membrane nor to the rise and fall of the endogenous

activity (Fig. 3.2.). However, the maximum at 64 hr. does correspond to the transition between logarithmic and stationary phases of the growth cycle.

CHAPTER IV

RESULTS II

THE PHYSICAL AND CHEMICAL PROPERTIES OF THE MEMBRANE FRACTIONS ISOLATED FROM HALOBACTERIUM SALINARIUM

4.1. Introduction

The nature of the lipid component of the membrane of H. salinarium, strain 1, has been investigated in fair detail (Chapter III). The aim of the work described in this chapter was therefore to investigate further the nature of the protein component of the membrane, primarily by amino acid analysis. Since the amino acid analysis of the total protein of a crude preparation is only of limited use, attempts were made to fractionate the membrane-rich particles by gel filtration. Later chapters are concerned with alternative fractionation procedures.

In the course of this work, it was discovered that a number of protein fragments could be split from the membrane fraction and these fragments are described. In addition, the physico-chemical properties of the crude membrane fraction (NM) and of the purified membrane fraction (R) have been investigated. The enzymological properties of the fractions are described in Chapter VII.

Electron micrographs of the membrane fractions are not available. For this reason, the methods of preparation of the cell envelope fragment bags and of the membranes (fraction NM) outlined in Steensland & Larsen (1969) and detailed in a private communication were followed with only a few minor modifications. These modifications concerned only the method of incubation of the crude membrane suspension with nucleases; Larsen and Steensland maintained their preparations at 2 mM Mg^{++} throughout and incubated at 37° instead of room temperature. It is hoped that the reader will accept the photographs in Steensland & Larsen (1969), Figs. 5, 8 and 9, as being representative of the preparations of the similar fractions which are described in this thesis.

4.2. Effect of magnesium ion on the membrane

The crude NM fraction was normally prepared and washed in a medium containing 2 mM MgCl_2 . At this concentration of magnesium, centrifuging at 150,000 g. (4 hr.) yielded a two-layered pellet, the lower layer being opaque and gelatinous whilst the upper layer was more transparent and mobile. On prolonged centrifuging a higher proportion of the lower layer was obtained. Increasing the concentration of magnesium in the suspending solution had the same effect. This observation suggests that the two fractions differ only in particle size; an increased concentration of magnesium favouring aggregation and a decreased concentration of magnesium favouring disaggregation. The two layers had identical cytochrome and carotenoid contents. They also had comparable buoyant densities (as measured in a sucrose density gradient), carotenoid contents and cytochrome contents (Table 4.1.).

Table 4.2. shows the effect of the duration of centrifuging at 150,000 g. on the proportion of protein sedimented as the lower, more dense layer. The effect of magnesium on the sedimentation pattern is shown in Table 4.3.

It is apparent from Table 4.3. that if centrifuging follows immediately upon resuspension in distilled water a considerable quantity of membranous material is recoverable in the lower layer whereas after some time in water some of the membrane is sedimentable as the upper layer. It thus appears that the Mg^{++} is bound loosely in some way to the lipoprotein structure and diffuses out relatively slowly.

As the upper layer was difficult to separate from the supernatant, the lower layer only was used for the preparation of R. A higher concentration of magnesium ($> 2 \text{ mM } \text{MgCl}_2$) would have increased the yield of lower layer, but would also increase contamination with amino sugar components from the envelope (Steensland & Larsen, 1969). The R fraction obtained from NM could be sedimented similarly as a two-layered pellet.

TABLE 4.1.

The effect of magnesium ion on the two layers
(upper and lower) of the sedimented membrane
fraction NM

The upper and lower layers of NM after centrifuging at 150,000 g (4 hr.). Cytochrome was measured by difference spectra (see section 2.9.). Density was measured by centrifugation (100,000 g for 24 hr. at 0°) in a sucrose density gradient (0 - 45%, w/w, sucrose) in the Spinco ultracentrifuge, rotor SW 50.

	<u>Upper Layer</u>	<u>Lower Layer</u>
Cytochrome (a.u./mg. protein)	0.115	0.111
Carotenoid (mg./g. protein)	8.4	7.2
Density (ρ) by resuspension of upper layer in Mg^{++} -free soln.	1.175 ± 0.010 (diffusely banded)	-
Density (ρ) by resuspension of lower layer in 10 mM MgCl_2	-	1.185 ± 0.005 (sharply banded)

TABLE 4.2.

The effect of magnesium ion on the two layers
(upper and lower) of the sedimented membrane
fraction NM (2)

<u>Duration of spin</u> <u>(hr.)</u>	<u>Upper layer protein</u> <u>% total protein</u>	<u>Lower layer protein</u> <u>% total protein</u>
3	50	50
4	37	63
6	25	75
8	12	88

TABLE 4.3.

**The effect of magnesium ion on the two layers
(upper and lower) of the sedimented membrane
fraction NM (3)**

Fractions were resuspended in a Potter Elvehjem homogeniser (5 - 10 passes by hand) and were centrifuged at 150,000 g (3 hr.).

	<u>% of total protein in lower layer</u>
upper layer resuspended in 2 mM Mg^{++}	50
upper layer resuspended in 20 mM Mg^{++}	100
lower layer resuspended in water respun immediately	80
lower layer resuspended in water dialysed against water for 36 hr.	0

4.3. Purification of the membrane-rich fraction NM

The sedimented nuclease-treated membrane contains 40% lipid, whose composition has already been described (section 3.4.), and 57% protein. The amino acid analysis of the fraction is presented (as Mole % only) in Table 4.4. together with a similar analysis of a red membrane fraction from H. halobium (Stoeckenius & Kunau, 1968).

In addition to a small amount of amino sugar (see below, section 4.6.) the fraction NM contains a small amount of ribonucleotide (equivalent to about 2 to 3% of the membrane dry weight). The orcinol reaction gave no indication of contaminating deoxyribonucleotide. The ribonucleotide was removed from the membranes by gel filtration on agarose (Bio-Gel A-50m) equilibrated with buffer β . No additional nucleotide was released during a second gel filtration step on columns of agarose equilibrated with either buffer β or buffered 2 M NaCl (Loeb, 1968).

A substantial amount of protein (36% of the total protein) was also released from the membrane on agarose gel filtration of NM. Both the protein and the nucleotide were eluted in the bed volume of the gel.

A slightly lower quantity of protein was released if 1 mM MgCl_2 was included in the elution buffer (23% total protein). The purified lipoprotein membrane fragments (R) were eluted in the void volume of both Bio-Gel A-150m and A-50m. Recovery of protein from the column was almost complete, provided that the crude nuclease-treated membranes (NM) were homogenised in distilled water (in a teflon Potter-Elvehjem homogeniser), and allowed to stand at 0° overnight. The protein released with the nucleotide was recovered free of nucleotide by ultrafiltration (Amicon Diaflo, filter UM-2, exclusion limit 1000 daltons), which removed most of the nucleotide, followed by gel filtration on Sephadex G-200 in buffer β when the purified protein is recovered in the void volume of the resin.

4.4. Characteristics of the protein fraction RP

RP contains no detectable lipid or cytochrome. Amino acid analyses of

RP fractions obtained with and without magnesium in the eluting buffer (RP₁ and RP₂ respectively) are presented in Table 4.4. The two analyses are very similar (15 residues within $\leq 1\%$, 2 residues within $\leq 2\%$) which may indicate a similar range of proteins.

The electrophoreses of the RP fractions were performed by Mr. F. Bellingham (see section 2.22.) on samples which had not been purified of nucleotide. Both RP₁ and RP₂ gave identical electrophoretic patterns confirming that they contain a similar range of proteins. The electrophoretic pattern obtained is presented diagrammatically in Fig. 4.1. Three of the five bands which stained for protein stained also for nucleotide. None of the bands stained for lipid.

4.5. Characteristics of the protein fractions P

No additional protein of type RP is released on passage of R a second time through a column of Agarose gel equilibrated with buffer β , or by subsequent passage through a column equilibrated in a buffer containing 2 M NaCl (see section 4.3.). However, a protein fraction, coded P₁, may be obtained from R fraction if it is passed through a column of Sephadex G-200 equilibrated in 0.05 M phosphate buffer, pH 7.0, containing 0.1 M NaCl and 10 mM NaF. The remainder of the lipoprotein membrane was eluted in the void volume of the column. The P fraction was eluted in the bed volume of the column (i.e. its molecular weight was < 3000) and accounted for approximately 18% of the total protein.

A similar protein fraction, coded P₂, was obtained by ultrafiltering a suspension of R in the Amicon Diaflo pressure ultrafiltration cell. The fraction P₂ passes through filter UM-1 (exclusion limit approximately 2000 daltons) but is retained by filter UM-2 (exclusion limit approximately 1000). The fraction constituted approximately 5% of the total protein. An amino acid analysis of fraction P₂ is presented in Table 4.5.

4.6. Characteristics of the lipoprotein fraction R

The membrane fraction R contains 49% protein, the amino acid composition of which is presented in Table 4.6., and 51% lipid whose composition has already been described (section 3.4.). Menaquinone (Vitamin K₂-8) was present at 1.1%

TABLE 4.4.

Amino acid analyses of fractions NM, RP₁ and RP₂
 isolated from H. salinarium and of a red pellet²
 isolated from H. halobium *

The methods used in amino acid analysis are detailed in sections 2.10 to 2.12.

In this table, analyses are presented only as Mole (%).

	<u>NM</u>	<u>red pellet*</u>	<u>RP₁</u>	<u>RP₂</u>
Lysine	2.5	2.1	3.0	3.3
Histidine	1.3	1.1	1.6	1.4
Ammonia	-	-	-	-
Arginine	3.0	4.0	3.4	4.1
Aspartic Acid	11.9	12.9	16.5	17.4
Threonine	7.7	8.1	8.1	8.5
Serine	7.3	6.8	10.4	9.0
Glutamic Acid	9.8	11.1	11.1	10.1
Proline	3.8	4.3	1.9	2.1
Glycine	10.4	9.5	10.2	9.0
Alanine	10.5	10.7	10.0	9.0
Half Cystine	(0.8)	(0.0)	(0.6)	(0.7)
Valine	9.5	8.2	7.4	7.4
Methionine	1.4	1.8	0.8	1.2
Isoleucine	4.3	4.6	3.7	4.6
Leucine	8.8	8.0	6.0	6.8
Tyrosine	2.2	2.9	3.0	2.7
Phenylalanine	3.2	3.3	2.5	2.7

* Stoeckenius & Kunau (1968)

FIG. 4.1.

**Polyacrylamide gel disc electrophoresis of fractions R and
RP derived from the membrane of *H. salinarium***

**The electrophoresis was performed in tris. HCl buffer at
pH 8.3 as described in section 2.22.**

Key :

L	stains for lipid ;	A	fraction R ;
P	stains for protein ;	B	fraction RP.
N	stains for nucleotide.		

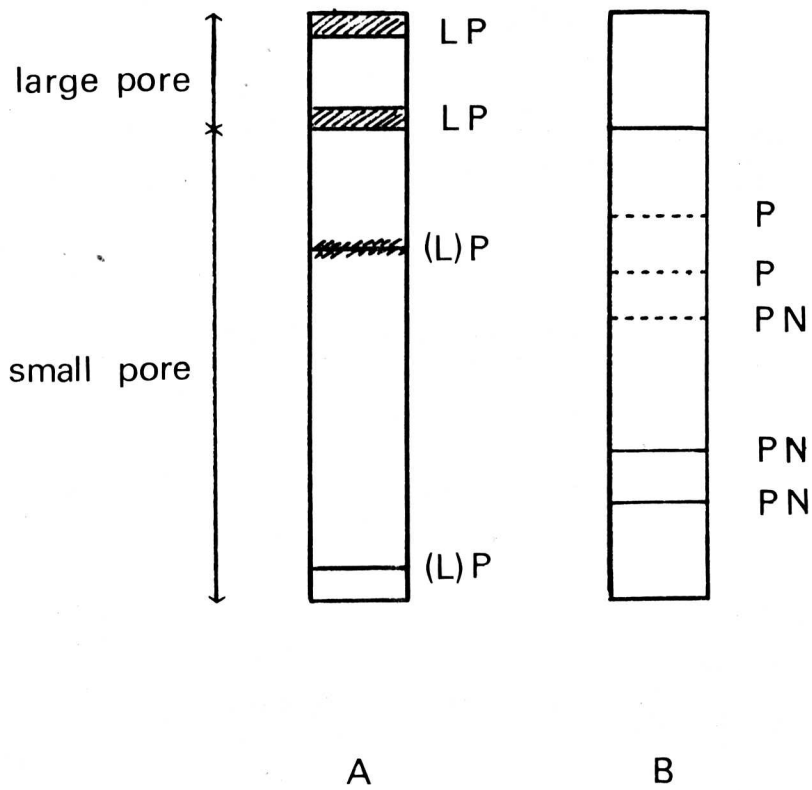


TABLE 4.5.

Amino acid composition of fraction P₂
isolated from H. salinarium membranes

The methods used in amino acid analysis are detailed in sections 2.10. to 2.12.

	<u>Anhydro amino acid/100 mg.</u> <u>salt-free dry weight</u>		
	<u>(mg.)</u>	<u>(μmole)</u>	<u>Mole (%)</u>
Lysine	6.5	51	5.5
Histidine	3.1	23	2.5
Ammonia	(9.0)	(530)	-
Arginine	2.0	13	1.4
Aspartic Acid	10.6	92	10.1
Threonine	6.8	67	7.1
Serine	5.9	68	8.5
Glutamic Acid	12.8	99	10.9
Proline	2.6	27	3.0
Glycine	7.4	130	14.2
Alanine	8.1	114	12.5
Half Cystine	(0.0)	(0)	(0.0)
Valine	5.0	51	5.6
Methionine	1.6	12	1.3
Isoleucine	3.3	29	4.3
Leucine	7.1	63	6.8
Tyrosine	4.2	26	2.9
Phenylalanine	5.6	38	4.2
TOTAL	93	913	100

and carotenoid at 0.36%. Both these compounds, together with the cell lipid are localised exclusively in the membrane fractions (Table 4.7.). Cytochromes a_1 , b_1 and c_5 were also present, and found only in membrane fractions. With dithionite as reducing agent, the typical difference spectrum of bacterial cytochrome b_1 was obtained. The presence of cytochromes a_1 and c_5 was suggested by using a preparation of H. salinarium, strain 1M, a mutant of strain 1, where the absence of carotenoid facilitated cytochrome estimation (see section 2.9.). It would be expected that cytochromes present in strain 1M would be present also in strain 1. A slight shoulder in the Soret peak in the dithionite-reduced difference spectrum of the wild type organism confirmed the presence of cytochrome a.

The absorption maxima for all three cytochromes are given in Table 4.8. The recovery of cytochrome in the envelope and membrane fractions is described in Table 4.9. RNA and DNA were not detectable in fraction R.

The membrane and envelope fractions were analysed for amino sugars in order to assess contamination of the membrane fractions with the amino sugar layer of the envelope (Steensland & Larsen, 1969). The analytical method is outlined in section 2.13. A number of variations in the hydrolytic procedure were tried, but the highest recovery of amino sugars was given in the method outlined in section 2.13. The method is basically that used by Steensland & Larsen (1969) except that the amino sugars were estimated by a modification of conventional amino acid analysis rather than by the colorimetric estimation of Randle and Morgan (1955). Glucosamine (4.2 ± 0.6 m μ moles / mg. protein) and traces of two unknown amino acids or amino sugars were detected in hydrolysates of whole cells, but not in hydrolysates of NM or R. The unknown (i) was eluted from the amino acid analyser in the same position as diaminopimelic acid, and the unknown (ii) in a position near to galactosamine. Both these compounds were present in NM, at concentrations of (i) = 3.6 m μ moles / mg. protein and (ii) = 1.6 m μ moles / mg. protein, together with smaller quantities of other unidentified compounds. None of these unknowns could be detected in hydrolysates of R. The possibility remains open that they are dipeptides or other products of the incomplete hydrolysis of protein. No attempt was made to confirm the presence of diaminopimelic acid by enzymic conversion to lysine. No muramic acid was detected.

TABLE 4.6.Amino acid composition of purified membrane fraction R

The methods used in amino acid analysis are detailed in sections 2.10. to 2.12.

	Anhydro amino acid/ 100 mg. salt-free dry weight		Mole (%)
	(mg.)	(μ mole)	
Lysine	1.36	10.6	2.3
Histidine	0.72	5.3	1.2
Ammonia	(0.77)	(45)	-
Arginine	2.04	13.1	2.8
Aspartic Acid	4.84	42.1	9.1
Threonine	3.34	33.0	7.2
Serine	2.40	27.6	6.0
Glutamic Acid	5.31	41.2	8.9
Proline	2.12	21.8	4.7
Glycine	2.83	49.6	10.7
Alanine	3.56	50.1	10.8
Half Cystine	(0.42)	(4.1)	0.9
Valine	4.79	48.4	10.5
Methionine	0.96	7.3	1.6
Isoleucine	2.28	20.2	4.3
Leucine	5.14	45.5	9.9
Tyrosine	1.48	9.1	2.0
Phenylalanine	2.40	16.3	3.5
Tryptophan	2.93	15.7	3.4
TOTAL	49	461	100

TABLE 4.7.

Recovery of menaquinone, carotenoid and lipid
in the membrane fractions NM, R and RU

	<u>Whole cells</u>	<u>NM^a</u>	<u>R^b</u>	<u>RU^c</u>
menaquinone, mg./g. protein	1.89	17.1	25.5	
total protein, g./l. culture	1.25	0.134	0.086	
total menaquinone, mg.	2.36	2.29	2.20	
% recovery	100	97	93	
carotenoid, mg./g. protein	0.53	4.85	7.5	
total protein, g./l. culture	1.25	0.134	0.086	
total carotenoid, mg.	0.66	0.65	0.65	
% recovery	100	98	98	
total lipid, mg./g. dry wt.	42	400	510	675
total dry wt., g./l. culture	2.5	0.235	0.176	0.158
total lipid, mg.	105	94	90	98
% recovery	100	90	84	93

^aNM, unpurified, nuclease-treated membrane-rich fraction

^bR, NM purified by passage through Agarose in buffer β

^cRU, R treated with urea, large lipoprotein fragment

TABLE 4.8.

Absorption spectra of the cytochromes

<u>Cytochrome</u>	<u>Source</u>	<u>Spectra λ max.</u>				<u>Ref.</u>
		<u>Reduced</u>			<u>Oxidised</u>	
		α	β	γ	γ	
a	bacteria	605		445		1
a ₁	bacteria	590				1
a ₂	bacteria	629				1
a ₃	bacteria	605		445		1
b	bacteria	564		430		1
b ₁	bacteria	560		430		1
c ₄	aerobic bacteria	551	522	414	414	2
c ₅	aerobic bacteria	555	524	418	414	2
a (592)	H.cutirubrum	592*		443		3
b (559)	H.cutirubrum	559		428		3
b (563)	H.cutirubrum	563		435		3
c (550)	H.cutirubrum	550				3
c (555)	H.cutirubrum	555				3
a	H.cutirubrum	606		443		4
b	H.cutirubrum	557		425		4
c	H.cutirubrum	553		423		4
o (b)	H.cutirubrum	559	525	428		4
b**	H.cutirubrum	561	532	431		4
A (a ₁)	H.salinarium	590		443		
B (b ₁)	H.salinarium	560	527	427	410	(5)
C (c ₅)	H.salinarium	554	524	418	410	

* absorption peak only in the presence of CN⁻

** dithionite reduced

1. Smith (1954)
2. Mahler & Cordes (1966)
3. Lanyi (1968)
4. Cheah (1969)
5. observed also by Bednar (1965)

TABLE 4.9.Cytochrome levels in the cell fractions

The method of assay for cytochrome and the definition of (a.u.) are described in section 2.9.

<u>Fraction</u>	<u>Cytochrome</u> (a.u./mg.protein)	<u>Total protein</u> (g./l.culture)	<u>Total cytochrome</u> (a.u.)	<u>% Recovery</u>
whole cells	0.0126	1.25	15.7	100
CEFB ^a	0.036	0.43	15.4	98
NM ^b	0.111	0.134	14.9	95
R ^c	0.156	0.086	13.6	86
RU ^d	0.160	0.060	9.7	62
RUP ^e	none	0.025	none	0

^aCEFB, cell envelope fragment bags

^bNM, unpurified, nuclease-treated membrane-rich fraction

^cR, NM purified by passage through agarose

^dRU, R treated with urea, large lipoprotein fragment

^eRUP, R treated with urea, small protein fragment

These results are similar to the observations of Steensland and Larsen (Larsen, 1967) who reported that the glucosamine present in the envelope was not retained in a lipoprotein membrane preparation similar to NM but that a 3-substituted amino sugar was retained quantitatively in the membrane. It would appear that if this 3-substituted amino sugar (which is similar, but not identical, to muramic acid) corresponds with any of the unknown peaks on the amino sugar chromatograms of NM (see above) then this compound is present in NM but not in R. Steensland (1967) had also reported that the whole of the amino sugar is released from NM on exhaustive dialysis against 1 mM EDTA, although only 80% or so of the amino sugar was released after dialysis against distilled water (Steensland & Larsen, 1969).

An estimate of the capacity of the R fraction to bind Mg^{++} was made using the technique of equilibrium dialysis (section 2.24.). On dialysis of R against 1 mM Na_2EDTA , virtually all the bound Mg^{++} is released ($< 0.01 \mu moles Mg^{++} / mg.$ protein remains bound). On equilibrium dialysis of the magnesium-depleted membrane (R) against increasing concentrations of $MgCl_2$, Mg^{++} is seen to become bound to the lipoprotein to a maximum of $0.35 \mu moles Mg^{++} / mg. protein$ (Fig. 4.2.). The Mg^{++} -depleted membrane may also be prepared by passage through a Sephadex G-200 column as used for the preparation of P_1 (see section 4.5.).

On agarose gel filtration, the fraction R, as normally prepared (i.e. containing some bound Mg^{++}) is eluted in the void volume of both Bio-Gel A-50m and Bio-Gel A150m, indicating a molecular weight in excess of 150 million daltons. The Mg^{++} -free fraction R had an apparent molecular weight of 30 million daltons.

The buoyant density of R was estimated by centrifuging to equilibration (100,000 g for 24 hr. at 0°) in a sucrose density gradient (25 - 45%, w/w, sucrose). Whether or not Mg^{++} -free, the R fraction exhibited a buoyant density (ρ) of 1.13 ± 0.01 .

The electrophoresis of R was performed by Mr. F. Bellingham as described in section 2.22. The electrophoretic pattern obtained for preparations of R fraction is represented diagrammatically in Fig. 4.1. The majority of the sample, including the carotenoid, remained at the top of the large or small pore

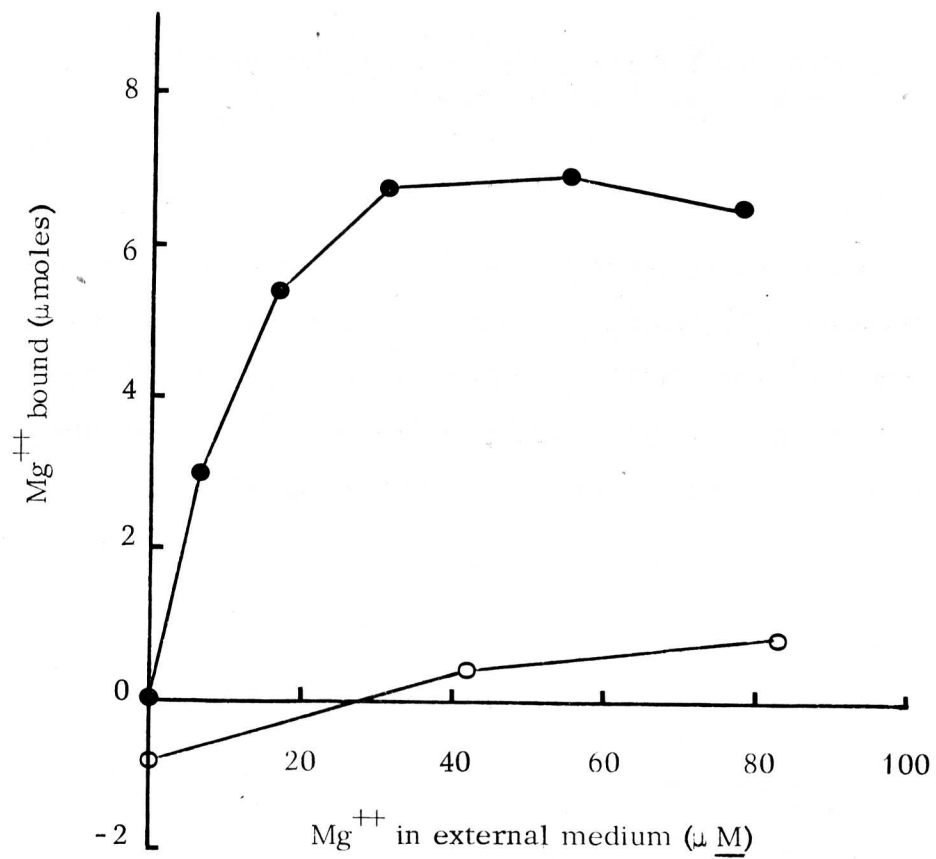
FIG. 4.2.

**Equilibrium dialysis of Mg^{++} depleted lipoprotein
membranes (R) against solution of $MgCl_2$**

20 mg. protein of magnesium-free fraction R were equilibrated against known quantities of magnesium. The bound magnesium was estimated by difference, by determining the magnesium remaining in the external solution by atomic absorption spectrophotometry. Fraction R was rendered Mg^{++} -free by exhaustive dialysis against EDTA.

Key :

- lipoprotein sample ;
- blank omitting lipoprotein.



sections. Two minor bands entered the gel ; one travelled with the migration front, a second was relatively slow-moving. Both bands stained for protein, and weakly for lipid. No trace of the bands characteristically present in the electrophoretic pattern of preparations of RP were observed.

The isoionic point of the membrane fraction R was determined as described in section 2.23. and was found to be at a pH of 4.1 ± 0.1 (average of three determinations).

4.7. Conclusion

The crude nuclease-treated membrane fraction (NM) and the purer membrane fraction (R) are both affected by magnesium ion. The difference, primarily, between the two layers found on sedimenting NM or R appears to be one of size rather than of composition. The gel filtration of R fractions in the presence and absence of magnesium ion also indicated a distinct size difference, which may be correlated with the observations of Steensland and Larsen (1969) in the electron microscope ; membrane sedimented in the presence of magnesium were considerably larger than those prepared in the absence of the ion.

This change of particle size, mediated by magnesium ion, in both NM and R is reversible and is not dependent on the presence of amino sugar components. Nevertheless, magnesium ion has a secondary function in the membrane in promoting the binding of this amino sugar component, or possibly, layer. It certainly seems to be the case that the successive removal of magnesium ion from the preparations of CEFB results in the successive removal of amino sugar components.

A third function of magnesium ion at the membrane level appears to be in promoting the binding of proteinaceous material, in particular the fractions RP and P (see sections 4.4. and 4.5.). Although the majority of the RP fraction (RP_1) could be obtained by gel filtration in the presence of 1 mM Mg^{++} , a greater proportion of apparently identical RP fraction could be obtained in the absence of the ion. As to the nature of the RP fraction, the evidence points to its being at least partly of ribosomal origin. In the electrophoresis of RP (plus nucleotide)

some of the bands observed stain both for protein and nucleotide although the overall amino acid analysis of the RP fraction is not particularly reminiscent of the analysis of soluble protein of ribosomal origin quoted by Bayley (1966).

The equilibrium dialysis experiments were designed to give a quantitative estimate of the degree of binding of magnesium ion to the membrane. Reliable estimates for the magnesium bound to membrane which had been washed several times in distilled water could not be obtained. The value obtained by the equilibrium dialysis method (see above) corresponds to approximately $0.049 \mu\text{moles Mg}^{++}/\text{mg. dry weight of CEFB}$. This value may be compared with Steensland & Larsen's value for the total amino sugar (Steensland & Larsen, 1969) of 1.3% or $0.073 \mu\text{moles}/\text{mg. dry weight of CEFB}$, assuming all the amino sugar to be glucosamine. Further, the phosphorus content of the membrane lipid is equivalent to $0.154 \mu\text{moles}/\text{mg. dry weight of CEFB}$. Assuming that the majority of this phosphorus is derived from lipids in which the phosphate groups are disubstituted (see section 3.4.), one might expect a stoichiometry of 0.5 moles of Mg^{++} bound per mole of phosphate, i.e. one magnesium ion bridging two lipid phosphate groups. The observed value for the bound magnesium is consistent with most of the phosphate groups participating in an ionic structure of this form.

The nature of the RP and P fractions will be more fully discussed (and compared with the RUP fractions) in Chapter VI. Similarly, the nature of the R fraction, e.g. in respect of its extremely hydrophobic character, is more profitably discussed in later chapters. Suffice it at this stage to say that the R fraction appears to correspond to the native plasma membrane, essentially free of cytoplasmic contaminants and of the outer layers of the cell envelope.

CHAPTER V

RESULTS III

THE SOLUBILISATION OF THE LIPOPROTEIN MEMBRANE (R) BY DETERGENTS

5.1. Introduction

Some progress has been made in fractionating the protein component of the cell membrane of *H. salinarium*, strain 1, into a part which remains associated with the membrane lipid on gel filtration and a part, which consists of a number of protein fragments, which is released from the bulk of the membrane (Chapter IV). Chapters V and VI describe the further fractionation of the lipoprotein complex, R, which is assumed to be a relatively pure plasma membrane preparation.

The classic approaches to the disaggregation of lipoprotein structures have been many and varied. One of the most powerful methods has been to treat the preparations with detergents (Razin, Morowitz & Terry, 1965 ; Butler, Smith & Grula, 1967) and this method forms the basis of the work described in this chapter. The use of protein denaturing agents has also proved effective, and the results of this section of the work are presented in Chapter VI. Milder techniques, such as ageing (freeze-thawing) and heat treatment, were found to be less effective at disaggregating the membrane. Heating the membrane at 80° for 30 min. had no apparent effect. Similarly, sonication was a relatively inefficient solubilising agent ; only about 10% of the membrane material was disaggregated to particles non-sedimentable at 150,000 g for 4 hr. after sonication for 5 min. In this respect, the membrane of *Halobacterium* is akin more to the *Mycoplasma* membrane than the bacterial protoplast (Razin, 1967) although the solubilised particles appeared to be homogeneous on gel filtration (particle weight approximately 40 million daltons).

5.2. General solubility properties of the membrane

First the general solubility properties of the membrane were investigated.

The membrane fraction, R, was found to be surprisingly soluble in organic solvents. The fraction is completely soluble in chloroform-methanol-water mixtures provided that the mixture is one phase. The fraction is soluble in acetone-water mixtures up to 50% (v/v) acetone.

Water is also a solubilising agent provided that the concentration of dissolved electrolytes is kept low. It was noted (section 4.7.) that the preparation of R exists as membrane sheets in the presence of magnesium ion but tends to disaggregate to lipoprotein particles if the magnesium ion is removed. On agarose gel filtration the sheets had a particle weight in excess of 150 million daltons; the magnesium-free particles had a particle weight of 30 million daltons. However, these weights were obtained in the presence of 100 mM NaCl under conditions where some reaggregation might have taken place. A very approximate value for the particle weight of R fraction in the absence of electrolytes (a sample dialysed against EDTA and washed several times with distilled water) was obtained from the time of its sedimentation in the ultracentrifuge. In the Spinco preparative centrifuge, using rotor 50, the preparation was completely sedimented in approximately 8 hr. indicating a molecular weight of approximately 1.5 million. This value was calculated from the nomogram in the Beckman instruction manual for the Spinco L-2 centrifuge, making appropriate corrections for the density of R ($\rho = 1.13$).

5.3. Disaggregation of the membrane in sodium dodecyl sulphate (SDS)

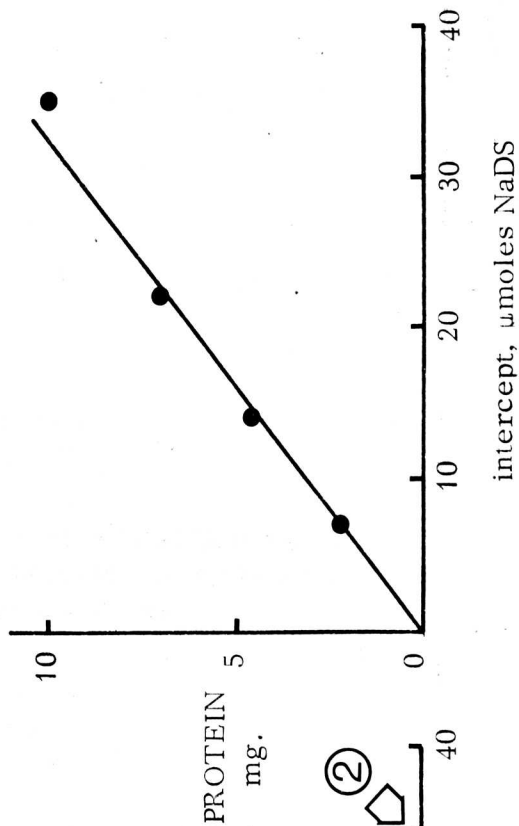
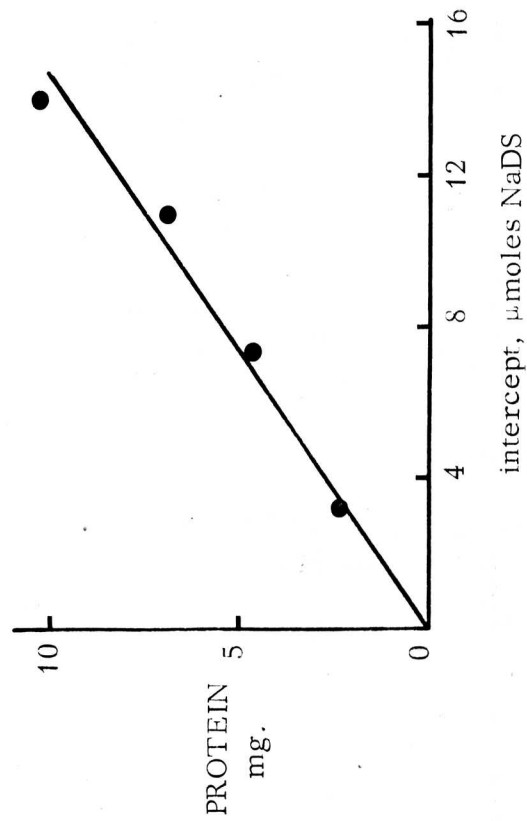
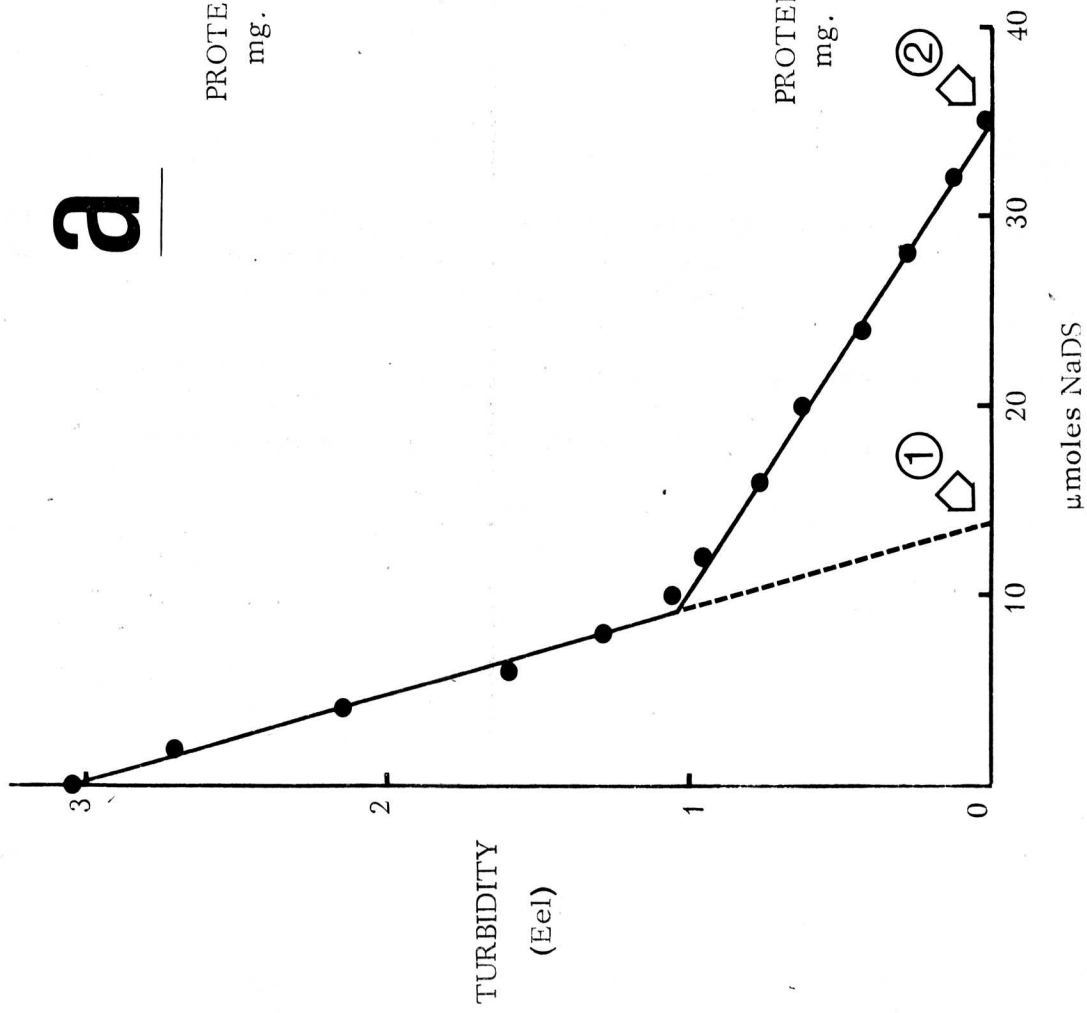
On adding SDS to a concentrated suspension of membrane fraction R (prepared in absence of magnesium) the suspension visibly clarifies. Quantitatively, the drop in turbidity (as measured on an Eel colorimeter, filter 608, 1.5 ml. micro tubes) appears to be stoichiometric with the detergent added. The relationship is biphasic and both linear regions of the curve (see Fig. 5.1.) may be extrapolated to zero turbidity when the intercepts are found to be proportional to the protein concentration used. These intercepts, labelled 1 and 2 in Fig. 5.1. may be plotted against protein concentration. These plots are straight lines, the

FIG. 5.1.

**The effect of sodium dodecyl sulphate (SDS) on the
turbidity of a suspension of the lipoprotein
membrane fraction R.**

Key :

- Curve a) :** Effect of increasing concentrations of SDS on 10 mg. (protein) of R. The total volume was 1.5 ml. Suspensions were made up in 5 mM NaCl, 0.25 mM tris. HCl, pH 8.0.
- Curve b) :** Intercept 1, as a function of protein concentration of R.
- Curve c) :** Intercept 2, as a function of protein concentration of R.



slopes of which represent the binding of SDS to lipoprotein material. At low detergent concentration (intercept 1) the slope of the curve gives a figure of 1.5 μ moles (0.412 mg.) SDS/mg. protein/ml. and at the high detergent concentration, 3.3 μ moles (0.95 mg.) SDS/mg. protein/ml.

5.4. Gel filtration of SDS-solubilised membrane

Membrane fraction R, solubilised in SDS as described in section 5.3. was subjected to gel filtration as described in section 2.21. Membrane (3 mg. protein/ml.) was suspended in detergent-free buffer or buffer containing 0.4 mM, 4 mM or 10 mM SDS (see section 2.21.). The agarose or Sephadex column used for the gel filtration was equilibrated in the same buffer as was used for the suspension of the sample. 2 ml. samples of the suspended or dissolved membrane were applied to the columns and the effluent was monitored for protein both on the Uvicord and by manual reading of $E_{280\text{nm}}$ on the Zeiss spectrophotometer. A summary of the fractions obtained at various detergent concentrations is presented in Table 5.1. The fractions are identified by their elution volume, V_e , compared to the void volume of the gel, V_o , for the fractionations on agarose, or by the function

$$K_{av} = \frac{V_e - V_o}{V_t - V_o} \quad \text{where } V_t \text{ is the total bed volume of the gel,}$$

for the fractionations on Sephadex.

All fractions obtained contained carotenoid, the ratio of $E_{500\text{nm}}$ to $E_{280\text{nm}}$ being constant.

Molecular weights were estimated after calibration of the gel columns with standards of known molecular weight (for columns run in the absence of detergent see section 2.21.). The results of these calibration runs are presented in Fig. 5.2. For the agarose columns no suitable standards were available except for the estimation of void volume (Blue Dextran). The calibration curve supplied by Bio-Rad was therefore used. This curve is reproduced in Fig. 5.3.

FIG. 5.2.

Calibration of Sephadex columns

Columns of Sephadex G-200 and G-100 were calibrated with proteins of known molecular weight as described in section 2.

Only one peptide was available for the calibration of the G-25 column.

The curve drawn is estimated from the expected exclusion limit for the gel, based on the results of Determann (1965) and the value (5000 m. w.) quoted for oligosaccharides by the makers of the gel (Sephadex booklet 2 ; Pharmacia, Sweden).

Key :

● G-200

■ G-100

△ G-25

◊^a estimated exclusion limit for G-25 gel.

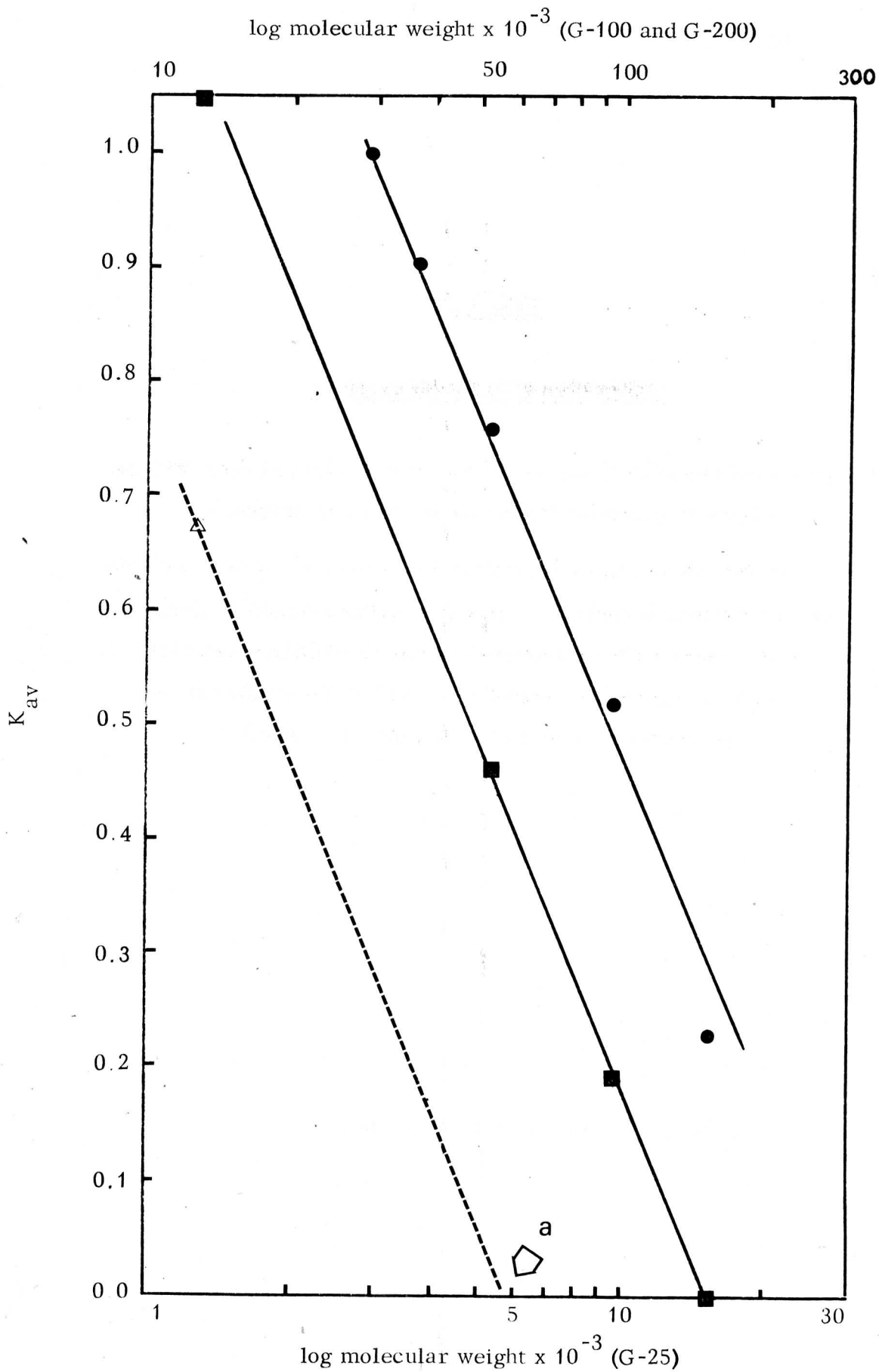


FIG. 5.3.

**Relationship between molecular weight and
elution volume on Bio-Gel A-50m**

(Reproduced from Bio-Rad leaflet BG-5E)

Key :

- a Human gamma globulin ;**
- b Bovine thyroglobulin ;**
- c Southern bean mosaic virus ;**
- d Tobacco mosaic virus.**

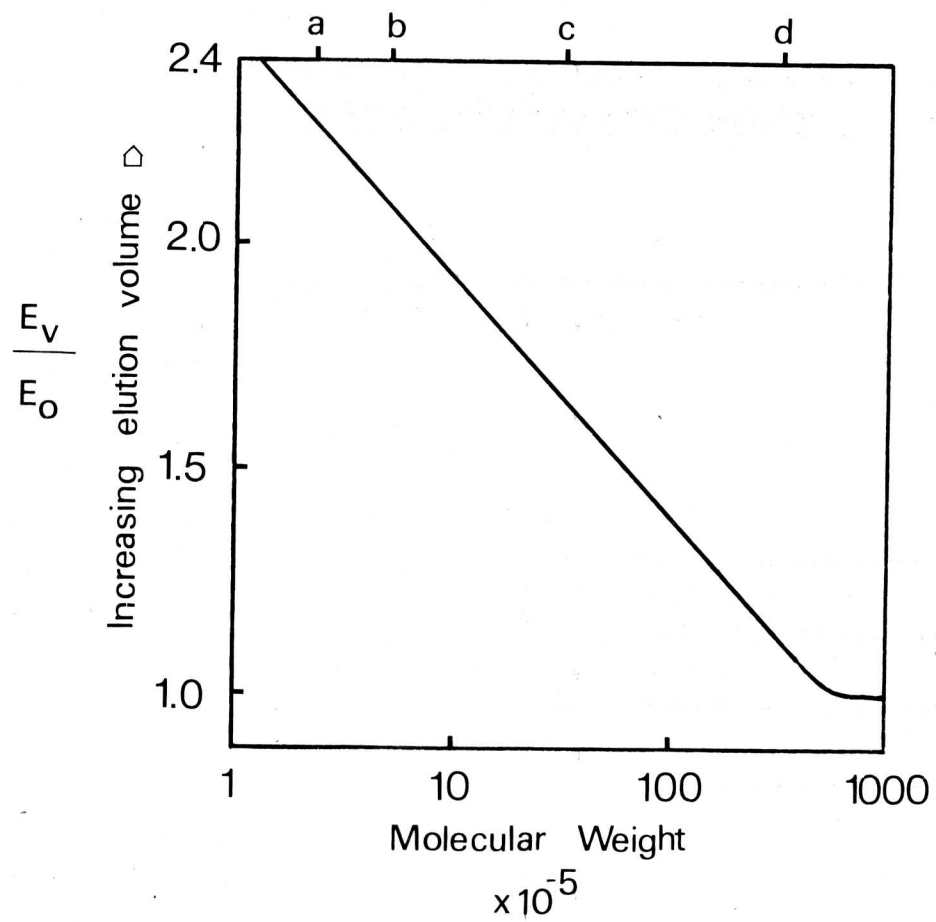


TABLE 5.1.

Gel filtration of SDS-solubilised membrane

Membrane fraction R was solubilised in SDS up to a concentration of 10 mM and was run through an Agarose or Sephadex column equilibrated in buffer containing SDS at the same concentration as in the sample. Protein was estimated as E_{280} and molecular weights were estimated from Figs. 5.4. and 5.5. E_v , E_o and K_{av} are defined in section 5.4.

A. Agarose gel filtration

The values in the main part of the table are protein, as a percentage of the applied sample.

	$\frac{E_v}{E_o}$	1.0	1.6	1.9	2.5
	molecular weight	>50 m	5 m	1.3 m	<150,000
SDS, mM					
none		100			
0.4		65	30	5	
4		7	1	92	
10		0	0	0	100

B. Sephadex gel filtration

The Agarose runs (A) in 10 mM SDS were repeated on Sephadex gels. All the sample applied was recovered in single peaks

	K_{av}	<u>molecular weight</u>
Sephadex G-100	0.1	120,000
Sephadex G-200	0.37	130,000

5.5. Density gradient centrifugation

Although the gel filtration experiments had suggested that the membrane dissolved in 10 mM SDS was homogeneous, it was nevertheless possible that separate protein-detergent and lipid-detergent complexes were present in solution but that these were not resolvable in this system. Attempts were therefore made to detect any such heterogeneity.

In a sucrose density gradient, 25 - 40% sucrose (w/w) in a 1:20 dilution of buffer β , it was possible to resolve three major bands. Two sharp bands at $\rho = 1.117$ and 1.120 contained carotenoid. A further diffuse band at $\rho = 1.134$ did not contain carotenoid but did contain the cytochrome system (as evidenced by its difference spectrum with dithionite as reducing agent). The density gradient run was performed in the Spinco centrifuge using rotor SW-50 operated at 100,000 g (60 hr.). The sample was layered onto the gradient in 10 mM SDS. There was no detergent present in the gradient.

Similar results were obtained on polyacrylamide disc electrophoresis of the solubilised fractions (see section 2.22.). In excess detergent, i.e. with detergent present in the gel, the whole sample travelled at the migration front. However, if a detergent-free gel was employed, samples dissolved in 10 mM SDS (for a protein concentration of 3 mg./ml.) split into a number of bands. A typical gel is represented diagrammatically in Fig. 5.4. The band at the migration front stained only for lipid, and contained carotenoid. The remaining bands stained strongly for protein but relatively weakly, or not at all, for lipid. Some of the protein bands contained cytochrome.

5.6. Reaggregation of the lipoprotein fraction R

On dialysis against changes of 0.002 M tris. HCl, pH 8.0 for 3 days at 0°, R fractions dissolved in 4 or 10 mM SDS partially reaggregated to a fraction of particle weight of approximately 20 million dalts (as estimated by gel filtration on agarose). If 10 mM Mg^{++} was included in the dialysis solution, a red lipoprotein fraction, completely excluded from Bio-Gel A-50m, was obtained. On centrifuging at 150,000 g for 4 hr. these reconstituted high molecular weight

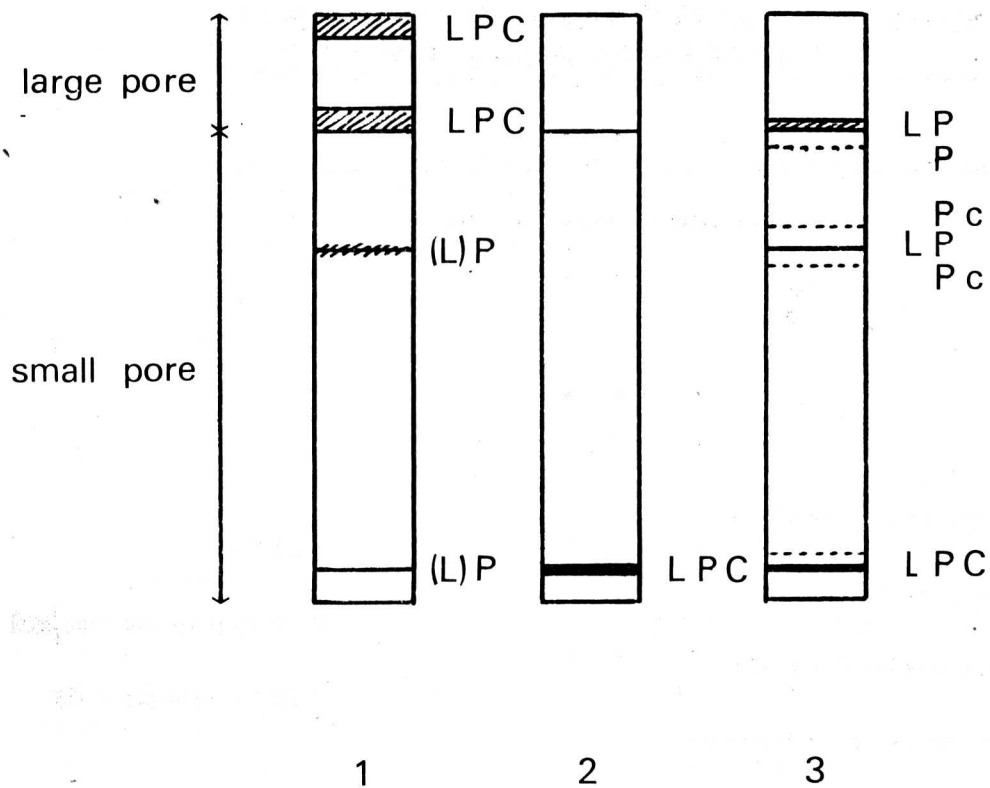
FIG. 5.4.

**Polyacrylamide gel disc electrophoresis of fraction R
in sodium dodecyl sulphate (SDS)**

**The electrophoresis was performed in tris. HCl buffer,
pH 8.3 as described in section 2.22.**

Key :

L	stains for lipid ;	1	no SDS ;
P	stains for protein ;	2	SDS in sample and gel ;
C	carotenoid visible ;	3	SDS in sample only
c	contains cytochrome.		



fractions gave pellets which were apparently identical to the original upper and lower layers respectively, obtained on centrifuging R. On density gradient centrifugation (100,000 g for 24 hr.) in sucrose gradients (25 - 45%, w/w, sucrose) both reconstituted fractions gave single bands at a density of $\rho = 1.13 \pm 0.01$.

5.7. Disaggregation of the membrane in multiple detergents

The effect of several detergents other than SDS on the membrane R were investigated. Sodium deoxycholate gave quantitatively similar results to SDS in the titration and gel filtration studies. Non-ionic detergents, such as Triton, had relatively little effect. In this connection, Steensland & Larsen (1969) had used Tween 80 and Triton X-100 to 'clean up' membranes, cytoplasmic contaminants being removed without apparent destruction of the membrane.

Of particular interest, though, was the effect of the combination of detergents (cholate, deoxycholate and SDS) used by Criddle, Bock, Green & Tisdale (1962) to dissolve mitochondrial membranes. These workers had isolated 'structural protein' by ammonium sulphate precipitation from a solution of mitochondrial electron transport particles in this detergent combination.

In the isolation of structural protein from H. salinarium membranes the basic method of Criddle *et. al.* (1962), method d, with the modifications and additional purification steps introduced by Lenaz, Haard, Silman & Green (1968), was followed. 20 mg. of fraction R (10 mg. protein) were completely solubilised by the addition of 20 mg. deoxycholate, 10 mg. of cholate and 7.5 mg. of SDS in buffer β . A sucrose density gradient centrifugation of the dissolved preparation demonstrated the separation of protein and lipid components. A carotenoid-containing band was obtained at $\rho \leq 1.10$, and a double band, containing cytochrome, was obtained at $\rho \sim 1.19$ in a gradient of 25 - 70% (w/w) sucrose. The conditions were otherwise as described in section 5.5. After the ammonium sulphate precipitation and purification a yield of structural protein (SP), 25% of the original protein was obtained. The amino acid analysis of the isolated material is given in Table 5.2. A comparison of this analysis with that of amino acids recovered from various SP preparations and from other protein fractions of H. salinarium is

presented in Table 5.3.

The extracted SP material exhibited the usual complete insolubility in aqueous media at neutral pH, although perhaps this is not surprising since the purification steps include heating in methanol at 50°. It contained no cytochrome or carotenoid, but showed a strong tendency to bind carotenoid and possibly other lipids. Attempts were made to demonstrate homogeneity, or otherwise, of the sample by electrophoresis in the phenol-acetic acid-urea system of Lenaz, Haard, Lauwers, Allman & Green (1968). The preparation apparently dissolved in this system but did not migrate in the gel.

5.8. Solubilisation of lipid-depleted membrane

If the lipid is removed from the membrane by extraction with chloroform-methanol (section 2.14.), the protein remains in solution in the upper (methanol-water) phase. On evaporation of the methanol under reduced pressure, the protein is denatured and precipitates. However, it may be dissolved in SDS. With gel filtration (see section 5.4.) on agarose in 4 mM SDS, a sample of 3 mg. protein/ml. gave a similar distribution of peaks to the native membrane (see Table 5.1.A) but the major peak was shifted to $\frac{E_v}{E_o} = 2.1$, which corresponds to a molecular weight of approximately 500,000.

On the other hand, an acetone extracted membrane (which would be expected to be depleted of only the non-polar lipids) gave a peak corresponding to that of the native membrane in 4 mM SDS.

5.9. Conclusion

The detergent, SDS, breaks up the fraction R into smaller particles. The biphasic nature of the disaggregation pattern seen in Fig. 5.1. may reflect two distinct disaggregation steps, as was also observed in the gel filtration analysis (Table 5.1.). Thus the initial disaggregation may be a breakage to particles of an apparent molecular weight of 1.3 million. Allowing for bound detergent, the particle has an apparent molecular weight of 1 million. There is also some evidence of polymeric forms, e.g. one at a molecular weight of 5 million (including detergent). The secondary disaggregation may reflect breakage to particles

TABLE 5.2.

Amino acid analysis of Structural Protein (SP)
isolated from H. salinarium membranes

The methods used in amino acid analysis are detailed in sections 2.10. to 2.12.

	<u>Anhydro amino acid/100 mg. salt-free dry weight</u>		
	<u>(mg.)</u>	<u>(μmole)</u>	<u>Mole (%)</u>
Lysine	3.5	27	3.0
Histidine	1.9	14	1.6
Ammonia	(2.3)	(135)	-
Arginine	7.2	46	5.1
Aspartic Acid	9.6	82	9.1
Threonine	5.3	52	5.8
Serine	5.0	58	6.5
Glutamic Acid	7.8	60	6.7
Proline	4.9	51	5.7
Glycine	4.4	77	8.6
Alanine	7.9	111	12.3
Cysteine	(0.0)	(0)	(0.0)
Valine	6.1	61	6.8
Methionine	3.2	24	2.6
Isoleucine	5.2	46	5.1
Leucine	13.2	117	13.0
Tyrosine	4.6	28	3.1
Phenylalanine	6.8	46	5.1
TOTAL	97.9	900	100

TABLE 5.3.

Comparison of amino acids recovered from various SP preparations and from various other protein fractions of *H. salinarium*

Protein	Source	Number of amino acid residues		
		Δ mole % < 1 ^a	> 1	> 2
SP ^b	HBHM ^c	10	7	4
SP ^d	<u>Neurospora crassa</u> mitochondria	10	7	4
SP ^e	Spinach chloroplast	4	13	6
envelope ^f	<u>H. salinarium</u>	7	10	8
R	<u>H. salinarium</u>	8	9	6
RU	<u>H. salinarium</u>	11	6	2
ribosomes ^g	<u>H. cutirubrum</u>	5	12	5

^a Mole per cent of a residue in the standard minus mole per cent of the same residue in the indicated protein. The standard is SP isolated from H. salinarium membrane-rich fraction.

^b Lenaz et. al. (1968)

^c HBHM, heavy beef heart mitochondria

^d Woodward & Munkries (1966)

^e Criddle & Park (1964)

^f Steensland & Larsen (1969)

^g Bayley (1966)

of apparent molecular weight 125,000 (approximately 85,000 allowing for bound detergent).

Whether these particles exist as lipoprotein complexes (with bound detergent) or as separate lipid-detergent and protein-detergent complexes is not entirely clear. The larger particle may possibly correspond with the particle obtained from the relatively slow dissolution of the membranes in distilled water, although the molecular weight of this particle (1.5 million) is probably only correct within an order of magnitude. However, if these two particles do correspond, it would indicate that the larger SDS particle was a lipoprotein complex.

Additional evidence is afforded by the gel filtration of lipid-free membrane in SDS, in which the solubilised particle appears to be approximately half the size of the corresponding particle from the native membrane. Relatively little success was achieved in the attempts to resolve the smaller SDS particle by either sucrose density gradient centrifugation or polyacrylamide gel disc electrophoresis when detergent was present in gradient or gel. However, it appeared that the protein and lipid components, as monitored by the cytochrome and carotenoid respectively, could be separated under conditions where excess detergent was removed, i.e. if detergent was omitted from the gradient or gel.

If the detergent was removed by exhaustive dialysis against distilled water, or 10 mM MgCl_2 , the membrane apparently reaggregated to a form similar to that of the original membrane. It was not possible to make a detailed comparison of the original membrane and the reconstituted membranes on a basis of protein composition, because an electrophoretic system which would separate the membranes into a reasonable number of their component proteins was not found. However, the lipoprotein unit obtained by dialysing the SDS-dissolved membrane against distilled water resembled the original magnesium-free preparation of R in molecular weight and buoyant density, and similarly for the preparations in the presence of magnesium ion. When reaggregated in either the presence or absence of magnesium, the reconstituted fraction showed no evidence of low molecular weight particles on gel filtration, indicating that there were no components present

in R which were not present in the reagggregates. On the other hand, gel electrophoresis of the reaggregate (prepared by removal of detergent by passage through a Sephadex column) redissolved in triple detergent did not correspond exactly to that of native R dissolved in triple detergent. In the reaggregate, the major bands were in the same positions as for native R, but the carotenoid was apparently present in the cytochrome-containing bands (F. Bellingham, private communication). However, the sample reconstituted by passage through Sephadex may not correspond with the sample reconstituted by dialysis.

CHAPTER VI

RESULTS III

THE FRACTIONATION OF THE LIPOPROTEIN MEMBRANE (R) IN UREA

6.1. Introduction

Following the experiments described in Chapter V, in which the effect of bifunctional molecules (primarily anionic detergents) on the membrane R was investigated, the study was extended to include an investigation on the effect of molecules which more specifically affected the hydrophobic interactions within the membrane. The obvious candidates are urea and guanidinium hydrochloride. It must be admitted, however, that the precise nature of the denaturing effect of these compounds is still uncertain. The action may possibly be a specific binding to groups on the protein (or lipid) with a weakening of covalent bonds responsible for structural integrity (Gordon & Jencks, 1963). Alternatively, the primary effect may be via a solvent denaturing effect, making water more hydrophobic and thus both affecting the hydrophobic bonding in the protein or lipoprotein molecule and increasing the solubility of hydrophobic molecules (Kauzmann, 1949; Scheraga, 1963). The reader is referred to Chapter VII for further discussions on possible solvent denaturing effects of various molecules (section 7.4.).

There was, however, a second reason for investigating the effect of urea and guanidinium chloride on the membrane fraction. In the course of work on the repeating base- and head-pieces of the mitochondrial inner membrane, Racker's group had observed that the head-pieces, which contained the adenosine triphosphatase, could be separated from the bulk membrane (TU particles) by treatment with trypsin and urea (Kagawa & Racker, 1965). More recently, Racker (1968) has shown that trypsin tended to damage the mitochondrial membrane, and has substituted a Sephadex filtration step for the original trypsin digestion. In this method, SU particles (the remaining membrane base-pieces)

and a soluble F_1 fraction are obtained. The F_1 fraction contained the adenosine triphosphatase, and other 'coupling factors' necessary for the complete restoration of oxidative phosphorylation in reconstituted particles.

In the experiments described below, agarose was used in preference to Sephadex and the gel filtration-urea treatment was performed in a one-stage process by passing R fraction through an agarose column equilibrated in urea.

6.2. The action of urea and guanidine on R fraction

On adding solid urea to a concentrated suspension of membrane fraction R in buffer β , the suspension visibly clarifies (Fig. 6.1.). Maximal clarity was obtained at urea concentrations in excess of 8 M, but because of the high viscosity of the resultant solution, the gel filtration and electrophoresis of the samples were normally performed in 6 M urea. At the lower urea concentration, the turbidity drop (as measured on an Eel colorimeter, filter 608, 1.5 ml. microtubes) was 91% of that observed in 8 M urea.

A qualitatively similar pattern was observed for the action of guanidinium chloride on R fraction.

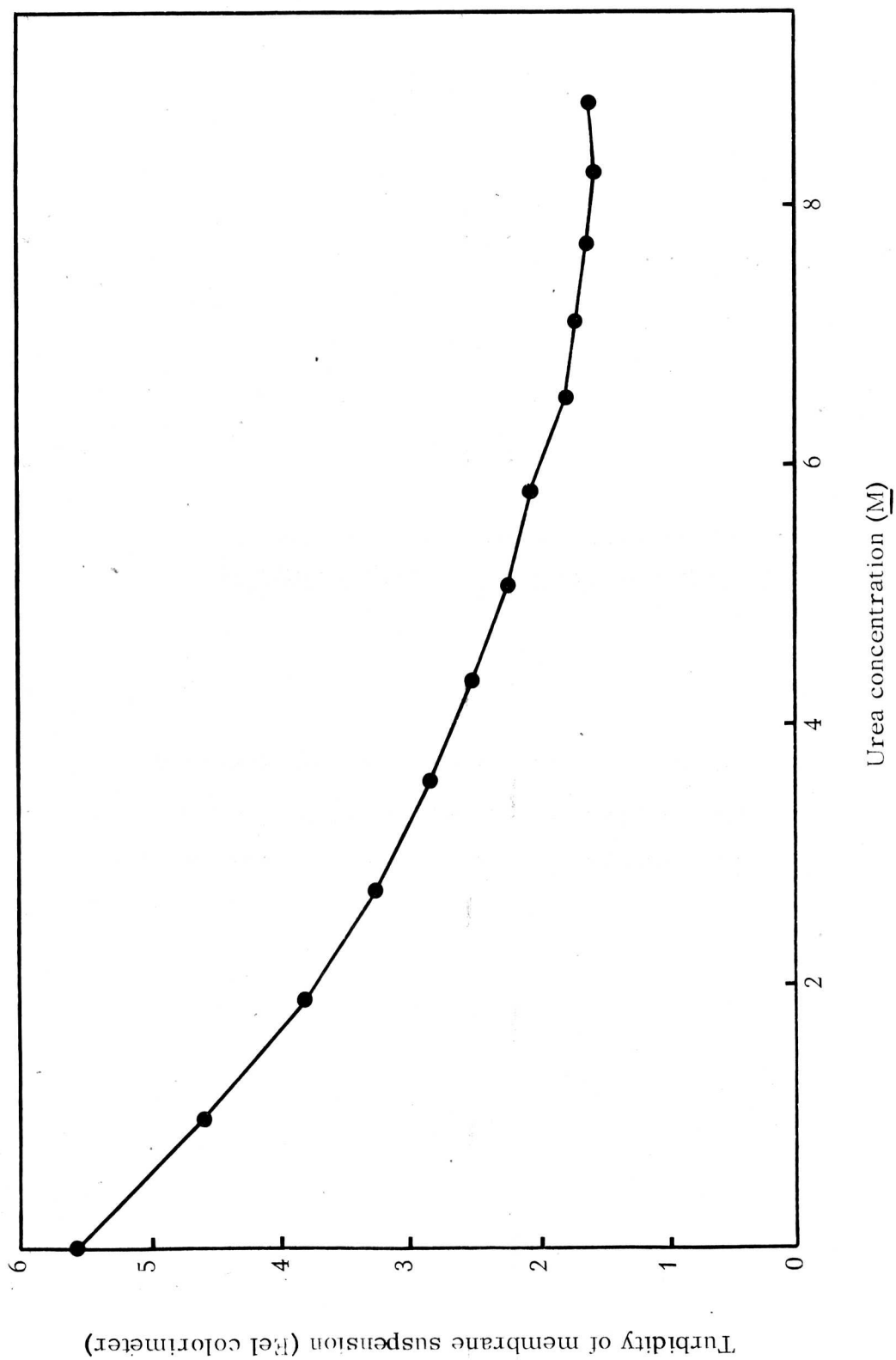
6.3. Gel filtration of urea-solubilised membrane

Solid urea was added to a concentrated suspension of R fraction in buffer β to a final concentration of 8 M. The sample was then applied to an agarose column (Bio-Gel A-50m) equilibrated in buffer β containing 6 M urea. After elution of the column with buffer β containing 6 M urea, a fraction was obtained in the void volume of the resin bed, which contained the majority, if not all, of the membrane lipid and cytochrome (see Tables 4.7. and 4.9.). This fraction was designated RU. A second fraction, designated RUP_1 , and containing about 30% of the total protein, was obtained in the fraction eluted at 1.95 times the void volume. This elution volume corresponds to a molecular weight of 800,000 in a urea-free gel (Fig. 5.3.). A third fraction, designated RUP_2 , and containing about 5% of the total protein, was obtained in the bed volume of the resin. This fraction was subjected to further gel filtration on Sephadex G-25 (fine) and was eluted in a single peak corresponding to a molecular weight of approximately 2300.

FIG. 6.1.

**The relationship between the turbidity of the
membrane suspension and urea concentration**

**Solid urea was added to a concentrated suspension of
membrane fraction R and the drop in turbidity followed
on an Eel colorimeter, 1.5 ml. microtubes, filter 608.**



Amino acid analyses of the three fractions, RU, RUP₁ and RUP₂ are presented in Tables 6.1., 6.2. and 6.3.

Gel filtration in urea of R fractions originally prepared in β buffer containing 1 mM magnesium gave a similar elution pattern to that of R fractions prepared in the absence of magnesium. However, if 1 mM MgCl₂ was included in the buffer for the gel filtration in urea, the release of RUP was suppressed to about 10% of the total protein.

6.4. Electrophoresis of urea-solubilised membrane

Solid urea was added to a concentrated suspension of R fraction in buffer β to a final concentration of 8 M. The sample was then subjected to polyacrylamide gel disc electrophoresis as described in section 2.22. 6 M urea was included in the gel and buffer solutions. After electrophoresis, two bands were observed, apart from material which remained at the top of the large or small pore sections. The two bands in the gel were in the same positions as in the electrophoresis of untreated R (Fig. 4.1.), but were more concentrated in comparison with the fraction excluded from the gel. The band at the migration front stained strongly for protein and weakly for lipid. The band just inside the small pore gel stained only for protein.

The band at the migration front was obtained in larger quantities by a scale-up of the analytical runs (section 2.22.). A molecular weight estimation on Sephadex G-100 indicated a value of 2000. An amino acid analysis closely resembled that of RUP₂.

6.5. Fractionation of guanidine-solubilised membrane

The action of guanidine on membranes was not examined as closely as that of urea, mainly because, perhaps incorrectly, it was assumed that guanidinium chloride would show the same qualitative effects as urea. However, one interesting observation is perhaps worthy of note. At one stage I was attempting to utilise centrifugation as an alternative to gel filtration in the isolation of RUP, and for comparison, I ran a sample of R fraction in 6 M guanidinium chloride instead of 6 M urea. Surprisingly, the tube set up its own density gradient (over a period

TABLE 6.1.

Amino acid analysis of fraction RU isolated
from *H. salinarium* membranes

The methods used in amino acid analysis are detailed in sections 2.10. to 2.12.

	Anhydro amino acid / 100 mg. salt-free dry weight		Mole (%)
	(mg.)	(μ mole)	
Lysine	0.9	7	2.1
Histidine	0.6	5	1.4
Ammonia	(0.9)	(50)	-
Arginine	1.9	12	3.6
Aspartic Acid	2.7	23	7.0
Threonine	2.9	29	8.6
Serine	2.0	23	6.8
Glutamic Acid	3.4	27	7.9
Proline	1.6	16	4.8
Glycine	2.0	34	10.4
Alanine	3.0	42	12.5
Cysteine	(0.2)	(2)	(0.6)
Valine	2.6	26	7.8
Methionine	1.0	8	2.3
Isoleucine	1.8	16	4.8
Leucine	4.5	40	11.9
Tyrosine	1.4	8	2.5
Phenylalanine	2.4	16	4.8
Tryptophan	(0.6)	(3)	(1.2)
TOTAL	35.5	328	100

TABLE 6.2.

Amino acid analysis of fraction RUP₁ isolated
from H. salinarium membranes

The methods used in amino acid analysis are detailed in sections 2.10. to 2.12.

	<u>Anhydro amino acid / 100 mg. salt-free dry weight</u>		<u>Mole (%)</u>
	<u>(mg.)</u>	<u>(μmole)</u>	
Lysine	2.3	18	2.0
Histidine	1.5	11	1.2
Ammonia	(2.0)	(117)	-
Arginine	3.6	23	2.5
Aspartic Acid	16.4	143	15.9
Threonine	8.7	86	9.6
Serine	6.3	72	8.0
Glutamic Acid	16.9	131	14.6
Proline	3.1	32	3.6
Glycine	5.3	93	10.3
Alanine	6.4	90	9.9
Half Cystine	(0.0)	(0)	(0.0)
Valine	4.7	47	5.2
Methionine	1.0	8	0.9
Isoleucine	4.3	38	4.2
Leucine	6.1	54	6.0
Tyrosine	5.4	33	3.6
Phenylalanine	3.4	23	2.5
TOTAL	97.4	922	100

TABLE 6.3.

Amino acid analysis of fraction RUP₂ isolated
from H. salinarium membranes

The methods used in amino acid analysis are detailed in sections 2.10. to 2.12.

	<u>Anhydro amino acid / 100 mg. salt-free dry weight</u>		<u>Mole (%)</u>
	<u>(mg.)</u>	<u>(μmole)</u>	
Lysine	7.3	57	6.2
Histidine	2.9	21	2.3
Ammonia	1.6	94	-
Arginine	2.8	18	2.0
Aspartic Acid	10.5	92	10.1
Threonine	6.2	62	6.7
Serine	8.3	95	10.4
Glutamic Acid	19.7	153	16.6
Proline	3.4	33	3.6
Glycine	8.3	146	15.8
Alanine	6.2	87	9.4
Half Cystine	(0.0)	(0)	(0.0)
Valine	4.0	40	4.4
Methionine	1.6	12	1.3
Isoleucine	3.7	33	3.6
Leucine	4.4	39	4.3
Tyrosine	2.4	15	1.6
Phenylalanine	2.6	18	2.0
TOTAL	95	913	100

of 4 hr. at 150,000 g.) and three main fractions were separated. The usual type of red pellet had sedimented at the bottom of the tube, but this was overlaid by a brown pellet, which could be separated from the red one quite cleanly. In addition, a red band was present about a third of the way up the tube, at a density of $\rho = 1.20$. This third fraction was particularly interesting in that a cytochrome difference spectrum, using dithionite as reducing agent, suggested a C-type cytochrome (γ_{reduced} at 420 nm., α_{reduced} at 554 nm.). On the other hand, the cytochrome present may be the usual b_1 species, with absorption maxima shifted owing to 'solvent effects.' The carotenoid spectrum is shifted approximately 4 nm. to the blue in the same fraction. The other fractions gave the usual b_1 -type cytochrome, when resuspended in deoxycholate.

6.6. Conclusion

In studying the effect of urea on the fraction R, it was found that a fraction RUP separated out on gel filtration through Bio-Gel A-50m and could further be separated into fractions RUP₁ and RUP₂ (apparent particle weights 1 million and 2000 daltons respectively). It was also observed that release of the RUP fractions was inhibited by magnesium ions, thus raising the possibility that there might be some similarity between the RUP fractions and the RP or P fractions. The RP fractions are released from NM maximally in the absence of magnesium ion. The P fractions are released from R under a variety of conditions but one of these is gel filtration in a magnesium-binding buffer.

RUP₁ was, however, considered a unique fraction on the basis of its molecular weight and the observation that bands characteristic of RP were not observed during polyacrylamide gel electrophoresis of R in urea. On the other hand, RUP₂ may well be a similar fraction to P on the basis of similar molecular weights, and amino acid analyses. In addition, the band identified as RUP₂ obtained in the acrylamide gel electrophoresis of R in urea (see section 6.4.) is also present in runs of untreated R.

It is possible therefore that RUP₂ is not uniquely released from R by urea but may also be released from R by the removal of residual magnesium ion.

CHAPTER VII

RESULTS V

ENZYMIC STUDIES

7.1. Introduction

In Chapters III to VI I have described the isolation and some of the physico-chemical properties of various membrane-rich fractions from H. salinarium. Of particular interest is the R fraction which may be the fraction, amongst those isolated, which corresponds most closely with the native plasma membrane.

However, a decision as to the precise origin of a given fraction, whether it is genuinely the plasma membrane or less than the total membrane (which is suggested by the high lipid content) or contaminated with material from the remainder of the envelope, is one that is very difficult to make without the additional evidence afforded by enzymic studies. It has been demonstrated that cytochrome and menaquinone are localised in the R fraction; it remains therefore to show that the NADH-menaquinone oxidoreductase, the menaquinone-cytochrome oxidoreductase, the cytochrome oxidase and possibly a succinate dehydrogenase are also associated with the membrane fraction. Adenosine triphosphatase might also be expected to be membrane-bound.

The presence of an NADH oxidase which was localised in a particular fraction (CEFB) and was halophilic (maximal activity in 1.7 M NaCl) has now been reported (Brown, 1966). Larsen (1967) made brief mention of an NADH dehydrogenase which displayed an extreme salt response (maximal activity in 4 M NaCl). Baxter & Gibbons (1956) reported a number of enzymes present in 'cell-free' extracts including a cytochrome oxidase (assayed with p-phenylenediamine as electron donor) which had a moderate salt response and a succinic dehydrogenase which had an extreme salt response. Stevenson & Brown (1967) reported the presence of a particulate adenosine triphosphatase which had an extreme salt response. Typical salt response curves are shown in Fig. 7.1.

FIG. 7.1.

**Effect of NaCl on the activities of enzymes
extracted from H. salinarium**

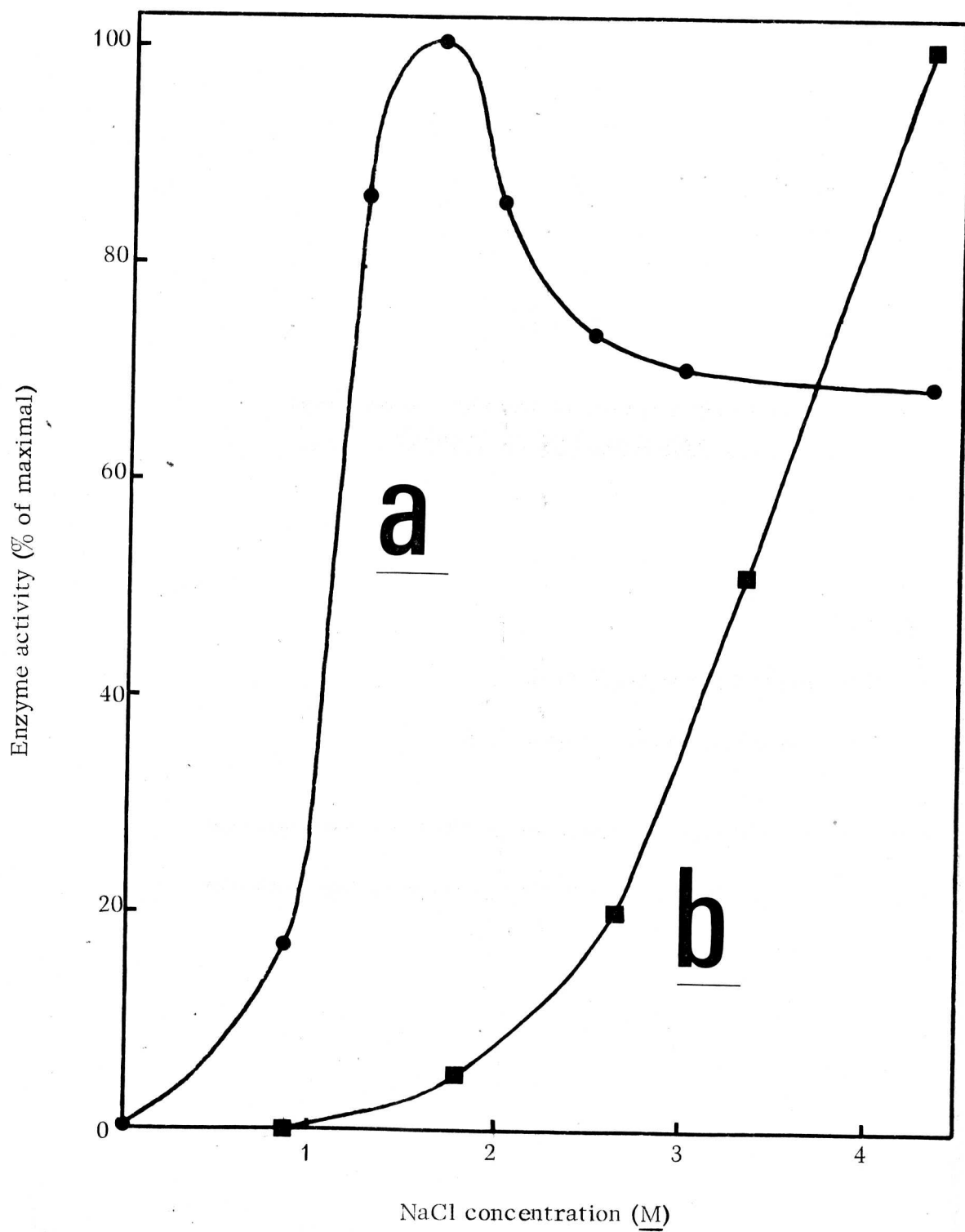
Key :

a : NADH oxidase (Brown, 1966) ;

b : NADH dehydrogenase (Larsen, 1967).

Curve a : typifies an enzyme with a moderate salt response ;

Curve b : typifies an enzyme with an extreme salt response.



It appears, therefore, that all the expected enzymes are present in particulate fractions, i.e. in CEFB. Baxter & Gibbons' cell-free extracts were obtained by centrifuging a suspension of broken cells at a relatively low speed under which a considerable quantity of particulate material remains in the supernatant; they did not assay the pellet. A more recent report (Cheah, 1969) describes the presence of a particulate cytochrome oxidase. A second general property of the enzymes is that they are halophilic, displaying minimal or zero activity in the absence of NaCl (see Fig. 7.1.). Thus no enzymic activity is expected, or obtained, for membrane preparations that have been exposed to distilled water during their preparation.

In order to investigate the distribution of enzymes between the membrane and other cell fractions, the deactivation of the enzymes in hypotonic media had to be avoided in some way. There are two possible approaches. Either one must attempt to preserve partial activity during the exposure to hypotonic media or one must accept the initial deactivation and attempt to reactivate the enzyme after exposure. The first approach is applicable to enzymes that retain a fair percentage of their activity in buffer α (i.e. 4M NaCl) after exposure to minimal amounts (say 100mM) of NaCl or $MgCl_2$. The second approach is applicable to certain enzymes that are reversibly deactivated on exposure to distilled water. Thus malate dehydrogenase, after it has been completely deactivated by exposure to distilled water, may be restored to 60% of its original activity by this method (Holmes & Halvorson, 1963). Ethanol dehydrogenase, on the other hand, was irreversibly deactivated on exposure to distilled water.

7.2. Localisation of adenosine triphosphatase

Stevenson (unpublished results, but alluded to in Stevenson & Brown, 1967) has successfully reactivated the adenosine triphosphatase after exposure to hypotonic media. A membrane fraction corresponding to fraction NM was prepared from whole cells, without going through the CEFB stage, by dialysing against 0.1M tris. HCl, pH 8.0 and, after treating with nucleases, centrifuging at 150,000 g. (3 hr.). If the pellet was immediately resuspended in buffer α , 30% of the original activity

(as measured on a crude sample of sonicated whole cells) could be restored. The remainder of the original total activity was present in the supernatant fraction. However, the specific activity of the restored enzyme was 2.6 times greater in the pellet than in the supernatant fraction.

7.3. Localisation of NADH oxidase

All attempts to reactivate the NADH oxidase after exposure to hypotonic media failed. It was therefore necessary to attempt to retain some of the enzymic activity in the CEFB by exposing to dilute solutions of stabilising cations (e.g. Na^+ and Mg^{++}) rather than exposing to distilled water (see section 7.1.). It was preferable to keep the concentrations of stabilising cations as low as possible, because these cations also promote the binding of other cell envelope constituents to the membrane.

Reference to Fig. 7.1. reveals that the activity of the NADH oxidase falls off rapidly below 1 M NaCl. Above this concentration of NaCl, the outer proteinaceous layers of the cell envelope are still fairly firmly bound. However, Mg^{++} ions are more efficient stabilising cations, and 70% of the maximal activity was retained when 100 mM MgCl_2 was substituted for the NaCl. Nevertheless, it was still possible that some of the outer layers of the cell envelope might still adhere to the membrane at this concentration of Mg^{++} . There remained the possibility that even lower concentrations of multivalent cations, e.g. spermine, might stabilise the enzyme without promoting the binding of other envelope constituents.

However, attempts to retain the activity of the CEFB NADH oxidase activity during exposure to hypotonic media containing up to 100 mM MgCl_2 , 33 mM spermine and/or 86 mM NaCl or KCl (in 0.1 M tris. HCl, pH 8.0) again failed.

7.4. Chaotropic agents

Whilst this work was in progress, an interesting paper was made available* (now published; Hatefi & Hanstein, 1969) which had important implications with respect to the nature of the NADH oxidase and its salt requirement. Hatefi & Hanstein had suggested that certain anions, notably SCN^- , ClO_4^- and I^- , and also

* as a private communication to Dr. D. E. Griffiths

certain molecules of the urea, guanidine type, increased the solubility of particulate proteins in water, whilst most common (alkali) salts tended to have the opposite effect. They gave appropriate thermodynamic explanations for their observations, a key factor in which was the observed lipophilic nature of membrane proteins.

Since the protein of R fraction is unusually lipophilic, it may be the case that water is 'chaotropic' with respect to NaCl solution, and that to maintain the membrane in an enzymically active conformation, either NaCl or a less chaotropic agent must be present. Since the original definition of 'chaotropic' (producing disorder) was applied to anions which tend to disorder DNA (Hamaguchi & Geiduschek, 1962) the obvious candidates are spermine, Mg^{++} and, to a lesser extent K^+ , as before!

However, Davis & Hatefi (unpublished communication) have shown further that chaotropic agents resolve the mitochondrial complex I (NADH-coenzyme Q reductase) and solubilise an NADH dehydrogenase of greatly enhanced electron transfer properties to 'artificial' electron acceptors, e.g. 2,6-dichlorophenolindophenol or ferricyanide. The effect of chaotropic ions on CEFB, described below, confirmed that a similar phenomenon could be observed with Halobacterium.

An assay was therefore devised for the ability of a membrane preparation to release the NADH dehydrogenase; even if, being partially denatured, the membrane preparation gave no NADH oxidase activity, it might still be capable of releasing the soluble enzyme.

7.5. The effect of chaotropic agents on cell envelope fragment bags

The ability of various chaotropic agents to release the NADH-ferricyanide oxidoreductase (ferricyanide reductase) from preparations of CEFB was investigated. The method of assay for the ferricyanide reductase is described in section 2.28. The results are presented in Table 7.1, together with the NADH-ferricyanide activities obtained for suspension of CEFB in 4 M NaCl, i.e. buffer α , ("Diaphorase activity") and in water, i.e. tris. buffer, for comparison.

a) The effect of the concentration of the chaotropic agent on the ferricyanide reductase activity

Perchlorate ion was chosen as the chaotropic agent for this investigation.

The method of assay for the ferricyanide reductase is described in section 2.28. The results are presented in Fig. 7.2.

b) The effect of preincubation of the CEFB with the chaotropic agent on the ferricyanide reductase activity

Aliquots of a preparation of CEFB were preincubated with 0.13 M or 2.7 M NaClO_4 in 0.1 M tris. HCl, pH 8.0 for 1 - 30 min. at 30° before assay of the ferricyanide reductase (see section 2.28.). The zero time values were obtained by adding the enzyme after the chaotropic agent. The results are presented in Fig. 7.3.

c) The effect of preincubation of the CEFB with NADH on the ferricyanide reductase activity

Aliquots of a preparation of CEFB were preincubated with 21 μM NADH in 0.1 M tris. HCl, pH 8.0 for 1 - 5 min. at 30° before assay of the ferricyanide reductase activity in the presence of 0.67 M NaClO_4 . The method of assay for the ferricyanide reductase is given in section 2.28. The results are presented in Table 7.2.

d) The effect of imidazole buffer on the ferricyanide reductase activity of CEFB

The assay for the ferricyanide reductase (chaotropically-released NADH-ferricyanide oxidoreductase) is given in section 2.28. When the normally used buffer (tris.) was replaced by imidazole at the same molarity and pH, a 29% increase in the observed activity was obtained.

e) NADH dehydrogenase activity with 2,6-dichlorophenolindophenol as artificial electron acceptor

A similar increase in the NADH dehydrogenase activity in the presence of chaotropic agent was observed when 2,6-dichlorophenolindophenol (50 mM) was used as electron acceptor, instead of ferricyanide. An increase of 10 times the "diaphorase" activity was observed in the presence of 2.7 M NaClO_4 .

TABLE 7.1.

The activity of the NADH-ferricyanide oxidoreductase
released from CEFB by chaotropic agents

Aliquots of a preparation of CEFB were added to an assay medium, as described in 'Methods', which included the chaotropic agent. The enzyme was added last. Specific activity is in units of μ mole NADH oxidised/min./mg. protein.

<u>Chaotropic agent</u>	<u>Specific activity</u>
(<u>4 M</u> NaCl)	0.65 ("Diaphorase" activity)
water	3.60
<u>2 M</u> NaClO ₄	35.0
<u>2 M</u> NaSCN	40.0
<u>2 M</u> urea	3.0

FIG. 7.2.

**The activity of the NADH-ferricyanide oxidoreductase
released from CEFB by perchlorate ion**

Aliquots of a preparation of CEFB were added to an assay medium, as described in 'Methods', which included sodium perchlorate at a concentration of up to 4 M. The enzyme was added last. Specific activity is in units of μ mole NADH oxidised/min. /mg. protein.

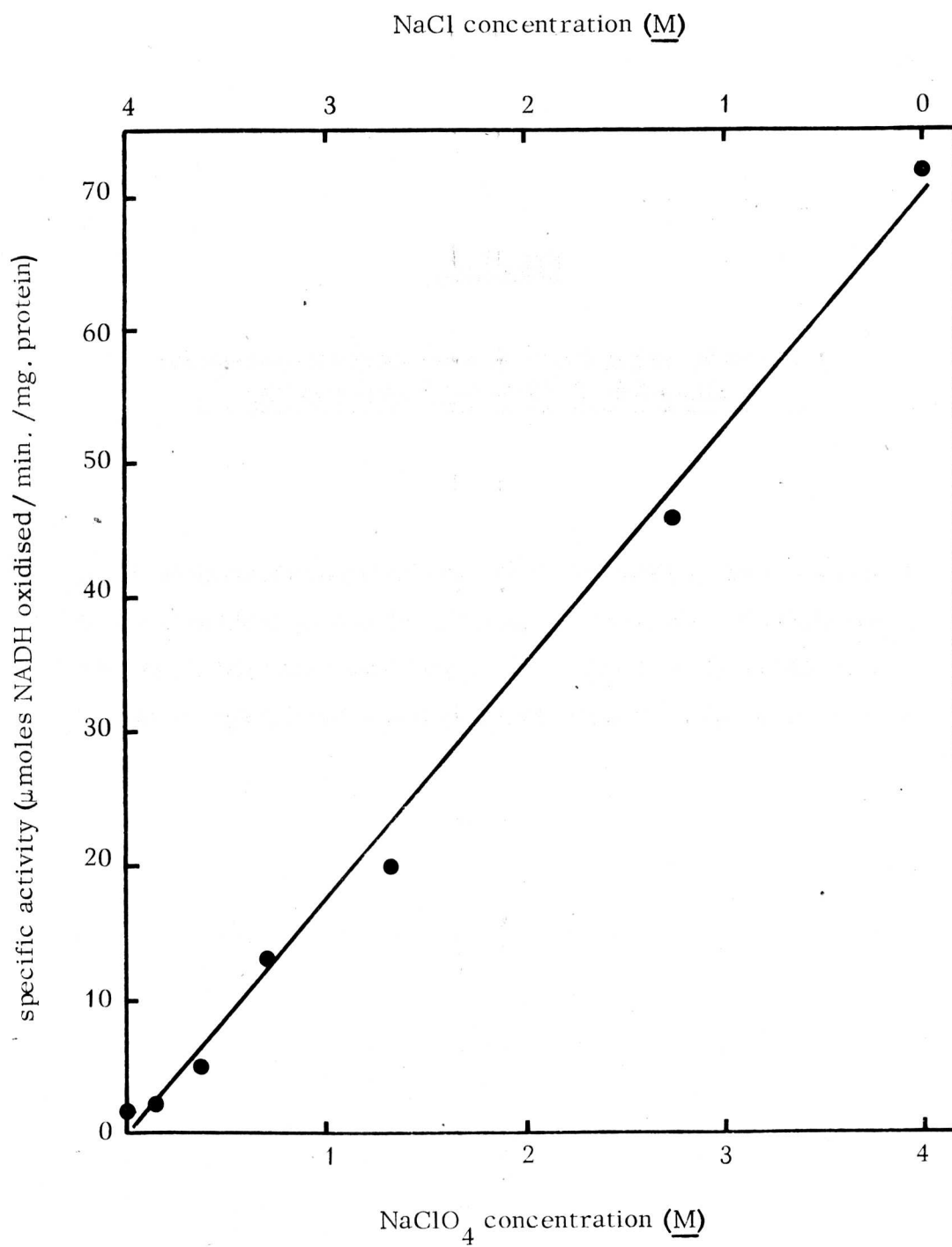


FIG. 7.3.

The release of ferricyanide reductase activity from CEFB as a function of time of incubation with the chaotropic agent

Aliquots of a preparation of CEFB were incubated with sodium perchlorate at a concentration of 0.13 M or 2.7 M before assay of the released ferricyanide reductase activity as described in section 2.28.

Key :

- 0.13 M NaClO₄
- 2.7 M NaClO₄

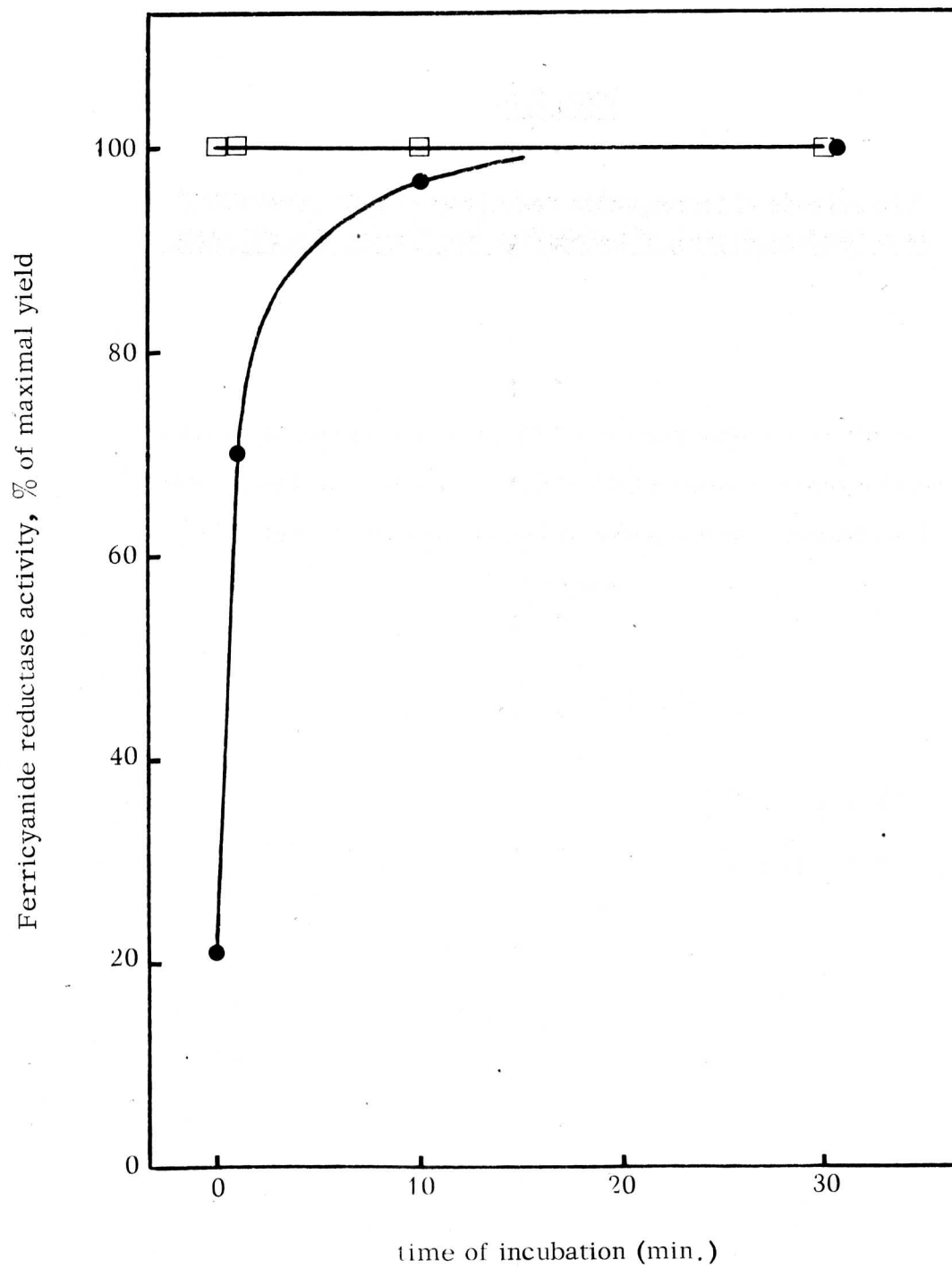


TABLE 7.2.

The activity of the NADH-ferricyanide oxidoreductase
released from CEFB by perchlorate ion

Effect of NADH

The CEFB were preincubated with NADH (21 μ M) for 1 - 5 min. before assay of the ferricyanide reductase. Specific activities are given as μ moles NADH oxidised/min./mg. protein.

<u>Preincubating medium</u>	<u>Ferricyanide reductase, Specific activity</u>		
	assayed without	assayed in 0.67 M NaClO ₄	
	NaClO ₄	preincubated	not preincubated
Buffer α (4 M NaCl)	1.5	1.5	12
0.1 M tris. HCl, pH 8.0	1.5	20	20

7.6. Incubation of cell envelope fragment bags with hypotonic media

In the first set of experiments, preparations of CEFB were diluted with hypotonic media, incubated at 0° for up to 14 hr. and then assayed for ferricyanide reductase activity. The general procedures are described in section 2.29. Results are presented in Fig. 7.4. Incubation at 30° instead of 0° was less successful in preserving reductase activity. Incubation in media containing iminazole buffer in place of tris, for which a modified assay system was used, gave similar results to those presented in Fig. 7.4. In the modified assay, iminazole buffer replaced the tris and NaSCN replaced the NaClO_4 . This combination increased the apparent specific activity of the reductase by 53%.

On the basis of the results presented in Fig. 7.4., an incubating medium of 0.1 M tris. HCl, pH 8.0 (or iminazole buffer in some experiments), 100 mM MgCl_2 and 3.3 mM spermine was selected as the best medium for preserving the ferricyanide reductase activity. Aliquots of a preparation of CEFB were incubated in this medium and centrifuged as described in section 2.29. The results of ferricyanide reductase activity of the pellets and supernatants obtained are presented in Table 7.3.

The third set of experiments were designed to determine whether preparations of CEFB, which had been incubated in a suitable hypotonic medium (see above) and had retained the ability to release an NADH-ferricyanide-oxido-reductase on treatment with a chaotropic agent, would still retain this ability after incubation with hypotonic media containing urea. Aliquots of a preparation of CEFB were first incubated in a medium containing 0.1 M tris. HCl, pH 8.0, 100 mM Mg^{++} and 3.3 mM spermine and centrifuged at 150,000 g (2 hr.). The pellet was then resuspended in a medium containing tris buffer, various concentrations of Mg^{++} and spermine, and 4 - 6 M urea. In all cases releasable NADH dehydrogenase activity dropped rapidly to zero in the second incubation, little activity remaining after 5 - 10 min. Slightly more success was experienced if imidazole buffer replaced the tris buffer in both incubations and in the assays, but only if the Mg^{++} concentration in the second incubation was extremely high (1 M).

FIG. 7.4.

The deactivation of NADH oxidase with time of
incubation in a hyptonic medium

Preparations of CEFB with NADH oxidase activity were incubated in hypotonic media as described in section 2.29. The integrity of the preparation was determined by assaying the preparation for chaotropically-induced ferricyanide reductase activity, as described in section 2.28., before and after incubation.

Key : Incubation mixes (section 2.29.) containing

- 100 mM Mg^{++} , 3.3 mM spermine ;
- 100 mM Mg^{++} , 0 mM spermine ;
- ▲ 66 mM Mg^{++} , 3.3 mM spermine ;
- 6.6 mM Mg^{++} , 33 mM spermine ;
- 0.66 mM Mg^{++} , 33 mM spermine ;
- △ 0 mM Mg^{++} , 33 mM spermine ;
- ◇ 0 mM Mg^{++} , 0 mM spermine.

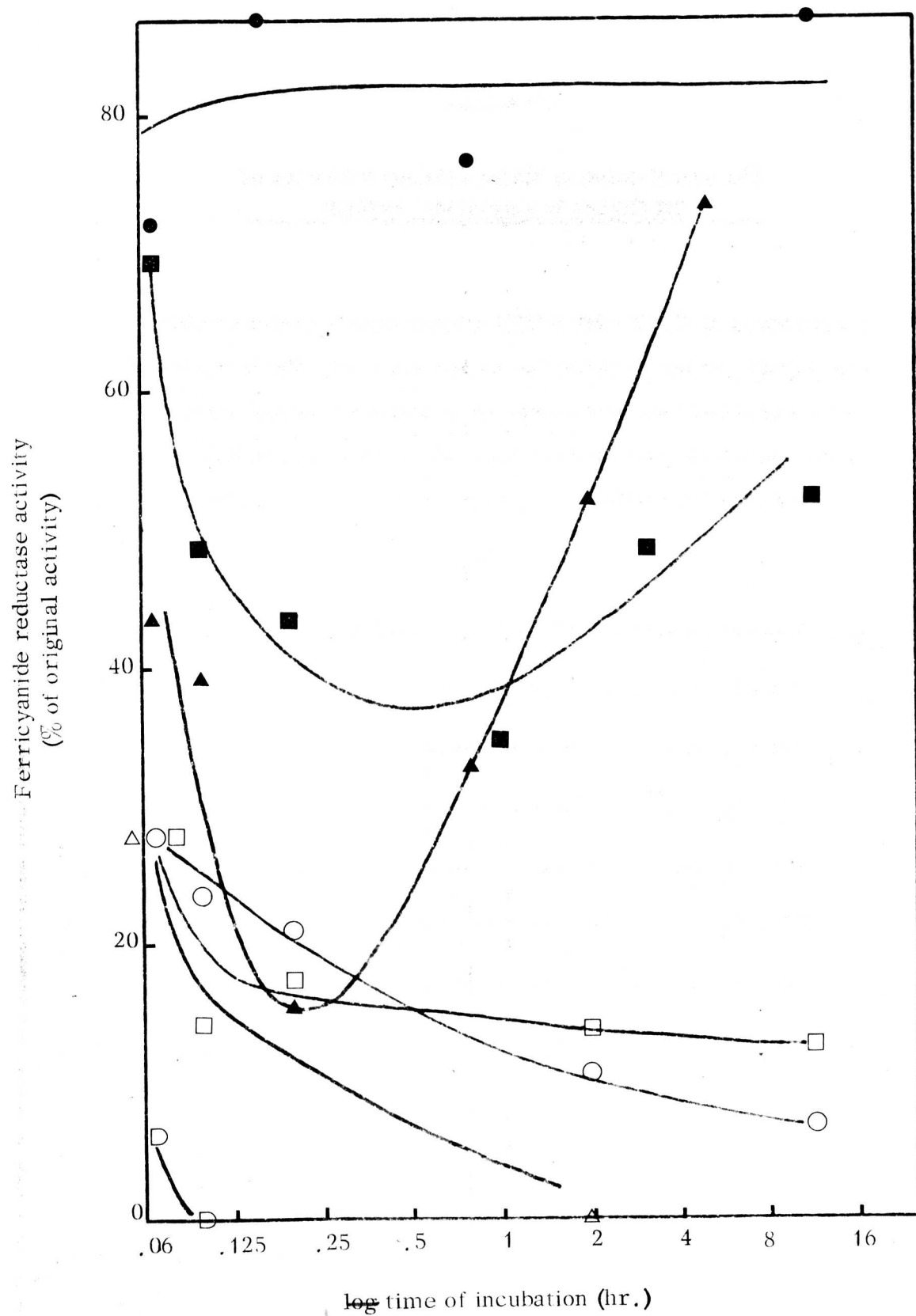


TABLE 7.3.

The activity of the NADH-ferricyanide oxidoreductase released by perchlorate of fractions derived from CEFB

Aliquots of a preparation of CEFB were diluted out with an incubation medium, and centrifuged, as described in section 7.6. Specific activity is in units of μ mole NADH oxidised/min./mg. protein.

<u>Dilution of CEFB (v/v)</u>	<u>[NaCl]</u>	<u>Specific activity</u>			<u>% recovery of original activity</u>
		<u>pellet</u>	<u>supernatant</u>	<u>total</u>	
-	4.3 <u>M</u>	-	-	58	(100)
1:50	86 <u>mM</u>	79	0	56	97
1:500	8.6 <u>mM</u>	57	0	40	70
1:125000	34 <u>μM</u>	38	0	27	46
1:30*	140 <u>mM</u>	94	0	65	112

* Incubated in 0.1 M tris. HCl, pH 8.0, 100 mM MgCl_2 (i.e. no spermine) and treated with nucleases at 0° before centrifuging.

Under these conditions (in the absence of spermine in the second incubation) about 15% of the original activity was retained over a period of 2 hr.

7.7. Conclusion

It is apparent from Table 7.1. that Hatefi's 'chaotropic agents' have an effect in Halobacterium similar to that observed in mitochondria. At the maximum concentration of chaotropic agent used (4 M NaClO_4) the 'native' ferricyanide reductase activity is stimulated a hundredfold. The activity of the released enzyme was proportional to the concentration of chaotropic agent used (Fig. 7.2.), although there was some evidence that at low concentrations of chaotropic agent, preincubation of the preparations of CEFB with the chaotropic agent at 30° stimulated the release of activity (Fig. 7.3.). Preincubation of the CEFB with NADH in buffer α prevented release of the ferricyanide reductase but preincubation of CEFB with NADH in salt-free buffer had no effect on the subsequent release of enzyme (Table 7.2.). These observations would indicate that a conformational change occurs in the CEFB NADH oxidase both on incubation with NADH and on transfer from a high salt to a salt-free environment. Possibly this second conformational change is itself chaotropically induced since (Fig. 7.2.) transfer of CEFB from buffer α to salt-free buffer released some ferricyanide reductase activity. Imidazole stimulates the activity of the ferricyanide reductase, just as it does the original CEFB NADH oxidase (Brown, 1966). This might possibly imply that histidine could be involved at the 'active sites' of both enzymes (or conformations of the same enzyme).

The attempts to preserve a near-native NADH oxidase during the exposure to hypotonic media at first sight appear to have succeeded. Table 7.3. strongly suggests that the NADH oxidase in Halobacterium is membrane-bound, like the adenosine triphosphatase (Stevenson & Brown, 1967). The relatively low recovery of total ferricyanide reductase activity on high dilution of the CEFB preparation (Table 7.3.) could be due either to a specific requirement for trace amounts of sodium ion for activity or loss into supernatant fractions. Possibly a slow solubilisation of the dehydrogenase has taken place.

However, it must be admitted that the pellet obtained after centrifuging preparations of CEFB exposed to hypotonic media (M) does not correspond to the pellet obtained in a normal membrane preparation (NM, see section 2.5.). The reason for this is the presence of a relatively high magnesium ion concentration, necessary for the maintenance of enzymic activity, in the medium from which M is obtained. It is significant to note that in all cases where ferri-cyanide reductase activity is preserved the suspension of M remains visibly turbid, as in the original suspension of CEFB. Loss of activity is associated with a clarification of the solution. This is true even with the preparation in 1 M Mg and 6 M urea, where only minimal retention of activity occurs. The obvious implication is that the active preparations are membrane bags whilst inactive ones are fragments. Possibly there is some cytoplasmic material present in M (trapped in the bags) just as there is in CEFB (Steensland & Larsen, 1969). In addition, the high Mg^{++} means that the amino sugar layer and any associated protein will still be bound but it may be presumed that the 'outer protein coat' has been dissolved (Steensland & Larsen, 1969). In preparing M from CEFB only 44% of the protein released in preparing NM from CEFB is obtained. The same percentage is obtained whether or not spermine is included in the diluting medium and whether or not the suspension is treated with nucleases.

Nevertheless, it is gratifying to find that the NADH oxidase is most likely to be localised either in the membrane NM or in the associated amino sugar layer.

CHAPTER VIII

DISCUSSION

The main conclusions from each section of this study have been presented at the end of each of the results chapters. It remains, however, to draw out some of the implications from these conclusions and to make a comparison between the results obtained and data, which have become available in the literature, from other workers in relevant fields of study.

In the study on ageing, the variations in cell and membrane composition that occurred during the growth cycle of Halobacterium were followed. As regards cell size, it has been suggested (Salle, 1967) that most bacteria tend to decrease in size during the growth cycle, and if the data of Dean & Hinshelwood (1966) may be taken to apply generally, it would appear that the maximal decrease occurs during the early logarithmic phase, immediately following a substantial increase during the lag phase. After this initial period of rapid change, only a slight decrease in cell size is noted. An important corollary from Dean & Hinshelwood's work was that the initial period of rapid change in cell size could be minimised by the use of a high inoculum. Possibly therefore, the apparent constancy of cell size during growth of Halobacterium is due to the high inoculum used and the associated absence of an observable lag phase.

The decrease in the proportion of membrane to whole cell dry weight during growth is particularly interesting since for most other bacteria which have been studied, a change in the opposite direction has been noted (Salton & Freer, 1965; Bodman & Welker, 1969; Shockman et al., 1963). However, the increase has been ascribed primarily to changes in protein levels, which again suggests that the membrane lipid component is relatively stable.

On the other hand, a paper by Marshall & Brown (1968) on the membrane lipids of H. halobium reports an increase in the proportion of membrane to whole cell during growth. Marshall & Brown (1968) also report large increases in the lipid content of the cell membrane and a significant change in the composition of

this lipid with the age of the culture - total lipid phosphorus more than doubled during the growth cycle. The discrepancy in the two sets of results may lie in the two methods of measuring cell bulk; Marshall & Brown used turbidity as a measure of cell mass, whereas I measured the salt-free dry weight directly. With an appropriate adjustment to the reference weight (i.e. of whole cells) a change in the proportion of protein to lipid in the membrane may be expressed either as an increase in lipid or as a decrease in protein.

In addition, it must be realised that whilst Marshall & Brown and I have been working with Halobacterium, the two species we have used are quite different. H.halobium has a much more complicated membrane structure, as pointed out in the introduction (Chapter I), and in particular there is some evidence that the proportion of intracytoplasmic membranes, which contain no lipid, increases markedly during growth (Stoeckenius & Rowen, 1967). There is also some evidence that the proportions of the purple membrane component and the gas vacuoles also increase during growth (private communication; C.F.W. McClare). Both these fractions (the intracytoplasmic membranes are probably collapsed gas vacuoles) would be expected to be present in the "membrane" preparation of Marshall & Brown (1968), together with some material derived from the cell wall (fraction I - Stoeckenius & Kunau, 1968). On the other hand, whilst the discrepancy in the gross membrane to whole cell or membrane protein to membrane lipid ratios in the two sets of results may be fairly easily overcome, Marshall & Brown's observation that the lipid composition changed during ageing is a little difficult to reconcile with my observations. Although the purple membrane component of H.halobium contains lipid, preliminary evidence suggests that its lipid composition is similar to that of the red fraction (Stoeckenius & Kunau, 1968).

It is of interest to see that in M.laidlawii a fall-off in protein synthesis has been observed as the cell culture ages which precedes that of lipid synthesis (Kahane & Razin, 1969). Similarly, it has been observed that in logarithmic cultures of E.coli cells, most of the phospholipids are stable and do not turn over (Kaufer & Kennedy, 1963). These results are consistent with the observed constant

lipid composition and the constant proportion of membrane lipid to cell dry weight in H. salinarium. Whether or not the membrane protein composition changed during ageing of the culture was not ascertained. However, Kahane & Razin (1969) had observed in M. laidlawii that there was no detectable difference in the turnover of various membrane proteins and Bodman & Welker (1969) found no significant variation in the membrane peptide amino acid composition with age of culture in B. stearothermophilus.

The changes in the levels of the carotenoid pigments with the age of the culture have been reported by Baxter (1960), working with H. salinarium, and have also been noted for H. halobium (Marshall & Brown, 1968). Baxter (1960) had observed that the levels of carotenoid varied with the age of the culture but not with the culturing conditions.

The apparently co-ordinate synthesis of carotenoid and menaquinone has not, to my knowledge, been observed before in Halobacterium. Certainly, there have been a large number of instances of changes in the levels of components of the electron transport chain during the transition between anaerobic and aerobic growth in facultative anaerobes (see section 3.1. and Wimpenny, 1969) and even of co-ordinate synthesis of some components. For instance, White (1965) observed in Haemophilus parainfluenzae that dimethyl vitamin K₂ and cytochrome b₁ were synthesised co-ordinately, and Frerman & White (1967) observed in Staphylococcus aureus that the vitamin K₂ and many of the major lipid components increased co-ordinately or near co-ordinately. However, these results do not seem to be directly comparable with Halobacterium. Although attempts have been made to grow H. salinarium anaerobically, these have failed and the organism must be regarded as an obligate aerobe. It could be supposed that the concentration of respiratory pigments might increase as a result of oxidative stress on the system. Certainly, the oxygen concentration in saturated salt solution is low, and oxygen uptake by the organisms might well at some stages be limited by the diffusion rate of oxygen. Experimentally, though, the levels of the carotenoid do not seem to be influenced by changes in the aeration of the medium (Baxter, 1960,

and confirmed by my own observations).

The maintenance of stoichiometry between the menaquinone and the carotenoid suggests that the two components are not only related biosynthetically but have a close relationship in the cell. It may well be that the carotenoid acts in a protective capacity on the menaquinone, preventing its destruction in u.v. light, as was originally suggested by Dundas & Larsen (1962, 1963). This would imply that the carotenoid was spatially located very close to the photo-activated system. Also, carotenoid pigments have been implicated in a protective capacity against photodynamic deactivation of NADH oxidase and succinate dehydrogenase (Mathews & Siström, 1960) and of adenosine triphosphatase (Rottem, Gottfried & Razin, 1968) in the presence of photosensitising agents and under aerobic conditions. Carotenoids have also been observed in complexes with the cytochromes (Mathews & Siström, 1959; Jackson & Lawton, 1958, 1959). However for H. salinarium such association is less likely since the cytochrome and carotenoid components may be obtained in separate bands on sucrose density gradient centrifugation of membrane fractions in detergents.

The high proportion of lipid in the membrane is worthy of note, and may reflect the highly hydrophobic nature of the membrane protein (see below). The proportion of lipid in the NM preparation (from a 3 day culture) of approximately 40% is comparable with that found in the membranes of other Gram-negative organisms (see Table 8.1.), but is higher than that of the average Gram-positive organism or of the mycoplasmas. The proportion of lipid in the R fraction is somewhat higher (50%).

The overall conclusion regarding the nature of the protein fragments RP, RUP and P (see Chapters IV and VI) is that part (primarily some or all of RP) is of ribosomal origin, and part (primarily RUP₂ or P and RUP₁) is derived from the plasma membrane.

Ribosomal contamination of membrane preparations has been noted by Coleman (1968) who observed in Bacillus amyloliquefaciens that the proportion of membrane-associated ribosomal material varies with the ionic strength of

TABLE 8.1.

The proportion of lipid in various bacterial
plasma membrane preparations

<u>Organism</u>	<u>Reference</u>	<u>% lipid in membrane</u>
Streptobacillus moniliformis, L-form	Razin & Boschwitz (1968)	40
Micrococcus denitrificans	Scholes & Smith (1968)	64
Escherichia coli	Miura & Mizushima (1968)	32
Halobacterium halobium	Stoeckenius & Kumau (1968)	c.40
Pseudomonas aeruginosa	Norton, Bulmer & Sokatch (1963)	35
Pseudomonas aeruginosa	Gray & Thurman (1967)	45
	average	43%
Gram-positive bacteria (16 species)	Salton (1967) average	24%
Mycoplasma (5 species)	Razin (1967) average	35%

the suspending medium. Similarly, Razin (1967) reports for Mycoplasma laidlawii that membrane-bound ribosomal material is dissociated during washing in magnesium-free buffer. Further, the small but not insignificant amounts of RNA or DNA that have been observed in the majority of membrane preparations from other bacteria are usually readily removed by treatment with nucleases.

These observations are consistent with the behaviour of the RP fractions from H. salinarium. However, it has not been established whether that part of the RP fractions which is of ribosomal origin becomes associated with the membrane during the isolation procedure, or whether it is genuinely membrane-bound. In both Bacillus megaterium (Schlessinger, 1964) and E. coli (Tani & Hendler, 1964) labile ribosome-membrane complexes have been implicated as the basic unit of protein synthesis. Protein synthesis is more rapid in ribosomes which retain their connection with the cytoplasmic or mesosomal membrane. Further, the stalk and head-pieces seen in electron micrographs of mitochondrial cristae and submitochondrial particles (Racker, 1967; Stiles & Crane, 1966) have also been seen in bacterial membranes (Abrams, Nielsen & Thaemert, 1964; Abram, 1965; Munoz, Freer, Eller & Salt, 1968; Biryuzova, Lukyanova, Gel'man & Oparin, 1964; Bladen, Nylen & Fitzgerald, 1964; Löw & Afzelius, 1964). Abrams et. al. (1964) have interpreted these stalked particles as attached ribosomes. Aronson (1966) reports that the ribosomes of B. megaterium are attached to the membrane by means of a polypeptide chain.

It is possible, therefore, that the ribosomal 'contamination' of the NM fraction is genuinely of membrane origin. Further, under the conditions of preparation of NM, the ribosomes might be expected to be completely disaggregated. Although no data are available on H. salinarium ribosomes, extensive work has been done on the ribosomes of H. cutirubrum (Bayley, 1966; Bayley & Kushner, 1964; Bayley & Griffiths, 1968a, 1968b; Griffiths & Bayley, 1969). The latter ribosomes are disaggregated in distilled water to particles which would not sediment in the ultracentrifuge, under the conditions of preparation of NM, unless bound to the membrane. Most of the nucleotide is, in fact, not sedimented with the membrane (after treatment with nucleases). The residual ribosomal material that remains

attached is therefore most probably genuinely membrane bound.

Again, with RUP_2 (or P) it is difficult to decide whether the fraction is part of the membrane or is a cytoplasmic contaminant; although probably the former is the case. The low apparent molecular weight (c.2300) is very unusual. Presumably the substance is a polypeptide of approximately 22 residues, although the amino acid analyses (RUP_2 and P_2) would suggest considerable heterogeneity in these fractions. (When the analyses are expressed as moles amino acid per 22 moles protein, they do not approximate well to whole numbers). The peptides appear to be unlike most of the other bacterial peptides that have been isolated (Waley, 1966) in that they contain no unusual amino acids. An alternative, since the fraction is released on treatment of the membrane with agarose (cf. Racker, 1967), is that the fraction corresponds to the low molecular weight mitochondrial adenosine triphosphatase inhibitor described by Pullman & Monroy (1963).

I have already mentioned the stalk and head-pieces seen in electron micrographs of some bacterial membranes. Although Abrams et.al. (1964) interpreted these as of ribosomal origin, other authors have suggested that they might correspond to or contain the membrane adenosine triphosphatase. (Abram, 1965; Munoz, Freer, Ellar & Salton, 1968). Both Abrams (1965) and Munoz et.al. (1968) report that an adenosine triphosphatase is released only after extensive washing of membrane preparations, and suggest that its release is dependent on the washing out of bound magnesium ions. An adenosine triphosphatase is released by osmotic shock from M.lysodeikticus, M.phlei, Azotobacter vinelandii and Corynebacterium creatovorans (Ishikawa & Lehninger, 1962; Brodie, 1959). Possibly the RUP_1 fraction corresponds to the H.salinarium adenosine triphosphatase. If so, it would appear to be the first time that urea has been used to release the bound enzyme from a membrane fraction in a bacterial system. It is, of course, a classic method for mitochondria (Kagawa & Racker, 1965; Racker, 1967, 1968). Although the apparent molecular weight of the RUP_1 fraction, in urea, is of the appropriate order (Myosin adenosine triphosphatase, molecular weight 520,000 - 600,000, Kielley, 1964; mitochondrial F_1 factor, molecular weight 284,000, Racker, 1967; adenosine triphosphatase released from S.faecalis, molecular

weight 350,000, Abrams & Baron, 1967, 1968) further enzymic characterisation is necessary to clarify the nature of the fractions (see section 7.2.).

From the studies on the solubilisation of R by SDS it is concluded that the solubilisation occurs in two major stages and it is suggested that there is an intermediate, possibly lipoprotein, particle which is present at low detergent concentrations, as well as the completely disaggregated particles, which are present at high detergent concentrations. The linear curves obtained on plotting the turbidity of the membrane suspension against detergent added suggest that the binding of SDS to lipoprotein is stoichiometric as has been demonstrated for proteins (Tanford, 1968). The biphasic pattern would indicate that two different conformational states of the membrane exist. Bovine serum albumin shows a similar two-stage binding pattern; 38 moles of detergent per mole of protein are bound at low detergent concentrations, 76 moles at high detergent concentrations (Decker & Foster, 1966). These observations are in marked contrast to those reported by Engleman, Terry & Morowitz (1966) who observed that the dissolution of *M.laidlawii* membranes proceeded via a continuum of states with no well-defined intermediates.

The secondary plots of intercepts against protein concentration (Fig. 5.1.) further support the suggestion that the binding of SDS to lipoprotein is stoichiometric, on the assumption that the binding sites have high binding constants (Tanford, 1968). On the other hand, Bont, Emmelot & Van Dias (1969), working with rat liver plasma membrane, report that the bound SDS is dependent on the concentration of free SDS, and exhibits an optimal value. In this connection, whilst the turbidity plots of R treated with SDS seem to suggest a 'titration' of R against SDS, the gel filtration studies support the suggestion that the type of disaggregated particle produced is a function of detergent concentration rather than absolute detergent quantity.

Whether or not the dissolved lipoprotein exists as a lipoprotein complex or as separate protein and lipid was not firmly established, but the evidence suggests that the larger (4 mM SDS) particle is lipoprotein and the smaller (10 mM SDS) particle is separate protein and lipid.

A number of reports of detergent-solubilised membrane preparations have

appeared (Korn, 1966, 1969) but there seems doubt as to the status of the complexes formed. Razin, Morowitz & Terry (1965) and Rodwell, Razin, Rottem & Argaman (1967), working with M.laidlawii, have been unable to demonstrate the separation of protein and lipid on density gradient centrifugation. On the other hand, Engleman, Terry & Morowitz (1967), working with M.laidlawii, Butler, Smith & Grula (1967), working with M.lysodeikticus, and Bont et.al. (1969), working with rat liver plasma membranes, have observed such separations. Further Bont et.al. (1969) have shown that the separation may persist after detergent has been removed by dialysis. Bont's observations are not paralleled in studies on the fraction R, although a similar phenomenon may be occurring in the density gradient and electrophoresis work in detergent-free buffers.

There is some evidence which suggests that the membranes may be re-constituted from the detergent solution and that the reconstituted material is similar to the original membrane. Similar observations have been made for the M.laidlawii, M.lysodeikticus and rat liver membrane preparations (Engleman & Morowitz, 1968; Butler, et.al., 1967; Bont et.al., 1969). However, it is emphasised that it is unlikely that the reconstituted material is identical to the original membrane, since it has been shown that considerable conformational changes occur when proteins are treated with detergents such as SDS (Tanford, 1968; Wetlaufer, 1962), and these may well not be reversible.

The use of the detergent sodium deoxycholate gave similar results to those of SDS, as did the triple detergent combination of SDS, deoxycholate and cholate. However, the detergent mixture was of additional interest in that its use enabled a 'structural protein' fraction to be isolated. Attempts to fractionate R dissolved in single detergents by the use of ammonium sulphate were not successful. The SP fraction is similar to other structural proteins that have been reported (Table 5.3.) and contains no detectable cytochrome or flavoprotein, as judged by its difference spectrum on reduction with dithionite.

The purified membrane fraction R contains the cell menaquinone and cytochrome and probably also the adenosine triphosphatase and NADH oxidase, in addition to or in association with structural protein. It may, of course, be an

oversimplification to suppose that the cell contains only one type of adenosine triphosphates or NADH oxidase; E.coli has been reported to contain several such enzymes of different specificities (Bragg & Hou, 1967). On the other hand, it is quite possible that the in vivo enzymes are unique and are membrane bound, the enzyme exhibiting different characteristics when solubilised.

Most of the enzymes from Halobacterium, which have been studied, require a high concentration of sodium ions for activity. In general, other ions can substitute for the sodium requirements, e.g. potassium at similar molarities or magnesium at lower molarities. In this particular study, the NADH oxidase of CEFB was found to be inactivated on exposure to 100 mM MgCl_2 . In contrast, an NADH oxidase reported by Hochstein & Dalton (1968a) was active in 100 mM MgCl_2 , the activity being 70% of the maximal activity observed in NaCl. This enzyme is reported as present in a cell-free extract from an extreme halophile.

Although the NADH oxidase of CEFB was inactivated on exposure to hypotonic media, the CEFB nevertheless showed ferricyanide reductase activity, after treatment with chaotropic agents, both before and after exposure to certain hypotonic media. For instance, the CEFB retained approximately 80% of their ability to release ferricyanide reductase activity, after treatment with chaotropic agents, after exposure to 100 mM Mg^{++} . It is suggested, therefore, that the NADH oxidase remains in a near-native state on exposure to 100 mM Mg^{++} .

The cytochrome oxidases reported by Cheah (1969) and Lanyi (1968) show a similar ion specificity; approximately 100 mM MgCl_2 substitutes well for 4 M NaCl in maintaining enzymic activity. In contrast, the adenosine triphosphatase is active only in the presence of a high concentration of either NaCl or KCl and though additional amounts of Mg^{++} stimulate this activity, the enzyme system is inactive in the absence of NaCl or KCl (Stevenson & Brown, 1967).

It is possible, therefore, that in the membrane in vivo, the respiratory chain is primarily stabilised by magnesium ions rather than sodium or potassium ions. Such a situation might occur if the membrane was shielded on both sides from the high concentration of monovalent ions present. The adenosine triphosphatase perhaps, is situated on the inner side of the membrane and is accessible to both

magnesium and potassium ions.

Although the enzymic experiments were designed primarily to monitor the influence of ion environment on the NADH oxidase, some interesting observations on the nature of the chaotropically released NADH dehydrogenase are perhaps worth mentioning. This enzyme is non-halophilic in the sense that it is more active in tris buffer than in buffer α , viz. essentially buffered 4 M NaCl plus 50 mM MgCl_2 . It is also stabilised by magnesium ions. Except at low concentrations of chaotropic agents, magnesium ions do not increase the observed activities of the ferricyanide reductase, but they do tend to diminish the rapid inactivation of the enzyme (as shown by a rapid drop from the initial rate of enzyme activity) which occurs in the absence of magnesium. If this enzyme is soluble, as might be supposed by analogy with Hatefi's work (unpublished, but see section 7.4.), then the properties of the enzyme serve as illustration of the profound changes in the properties of a particulate enzyme which may occur on solubilisation.

The NADH dehydrogenase has been studied also by Hochstein & Dalton (1968b) who used 2,6-dichlorophenolindophenol as electron acceptor. Essentially the study was on the diaphorase activity; chaotropic agents, other than water, were not used. It is of interest, however, that these workers observed that magnesium ions, spermine and polylysine stabilised the enzyme in the absence of sodium chloride.

The amino acid analyses of protein fractions, whilst originally intended as markers for protein fractions, have proved of additional interest in that they demonstrate the hydrophobic character of many of the fractions obtained, in particular R, RU and SP. It has been observed before that lipoproteins and particularly membraneous lipoproteins have a low ratio of hydrophilic to apolar amino acid residues (Hatch & Bruce, 1968). Also, Salton (1967) has suggested that it may well be a general feature of bacterial membranes that they contain highly hydrophobic protein, either enzymic or structural, although at the time of Salton's review, data was available only for membranes from Gram-positive cells and Mycoplasma. The membrane fraction of Halobacterium seems to conform to these observations, indeed the R fraction protein is more hydrophobic than the average membraneous lipoprotein on a basis of polar-apolar residue

count (Hatch & Bruce, 1968) which is quite surprising, since analysis of other Halobacterium proteins, e.g. of H. cutirubrum ribosomes (Bayley, 1966) or the outer protein coat component of H. halobium (Stoeckenius & Kunau, 1968) reveal a particularly high content of polar residues, especially aspartic and glutamic acids, an observations that has many times been connected with the halophilic properties of Halobacterium cells.

As I have already suggested, it is probable that the respiratory chain of the membrane in vivo is shielded on both sides from the highly ionic environment. The exterior of the membrane is overlaid by an amino sugar layer and an acidic protein coat. Part of the membrane itself (perhaps the adenosine triphosphatase) may form the shield on the inside, and possibly the membrane is shielded on the inside also by a ribosomal layer, corresponding perhaps with the acidic RP fraction.

The classic explanation for the solubilisation of the envelope of Halobacterium in water has been based on the observation that the cell protein is predominantly acidic. In the absence of high concentrations of alkali salts, the carboxyl anions are no longer electrostatically screened, and the envelope expands and dis-aggregates (Brown, 1963; Kushner & Bayley, 1963). Halophilic properties may be conferred on marine bacteria by succinylating free amino groups on the cell surface (Brown, 1964). The membrane of H. salinarium, however, is much more resistant to solubilisation when suspended in water than is the whole cell or intact envelope and, as might be expected, the membrane is much less acidic than is the envelope.

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